Using Design of Experiments to Improve a Batch Chemical Process

By

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B.S. Chemical Engineering, University of Florida, 2005

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Abstract

Novartis Vaccines and Diagnostics has made a strong commitment to manufacturing seasonal influenza vaccines through their cell culture technology called Optaflu®. The goal of this project is to improve overall process yield by modifying the upstream process. The focus is on using a batch process to generate a high-density cell culture and then infecting said culture. This thesis presents the approach of using a Design of Experiment series to change a manufacturing process.

Current vaccine production occurs with a fed-batch process by feeding glucose as a carbon-energy source for the final cell expansion step. This cell culture is diluted, infected, harvested, and purified for use in an influenza vaccine. Primarily, the project aims to increase cell density, using a batch process, at the infection step which should improve overall process yield. The project can therefore be broken into two main steps: batch cell growth and high-density infection.

Experiments for this project were conducted with a small-scale laboratory process that mimics the production process. The planned approach was a Design of Experiment series to screen parameters and partially optimize the cell growth process, a scale-up cell growth experiment, and finally another Design of Experiment series to explore high-density cell infection. While initial small-scale experiments showed extremely positive results, the results were not consistent and could not be replicated at a larger scale. A number of exploratory experiments were run to attempt to identify which factors inhibit high-density cell growth, particularly around scale-up, but no key parameter was identified. Given the process improvement and cost savings implications from the success of the initial small-scale experiments, this project is worth further exploration.

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List of Abbreviations and Definitions

Table 1: Abbreviations and Definitions

Acronym	Phrase
V&D	Vaccines and Diagnostics
FCC	Flu Cell Culture
DOE	Design of Experiment
MDCK	Madin-Darby Canine Kidney
CDM	Chemical Defined Media
WHO	World Health Organization
SRD	Single radial immuno-diffusion

Chapter 1: Introduction

This thesis covers the research conducted during a six month internship within the V&D division of Novartis AG between June and December of 2009. This project was sponsored by the Vaccine division's Technology Development group and the objective was to evaluate the feasibility of infecting high-density cell cultures made from a batch procedure. Included in this thesis is a series of experiments to test the feasibility of batch cell growth for high-density cell infection and an analysis of the experiment results.

1.1 Problem Statement

Novartis V&D has made a strong commitment to manufacturing seasonal influenza vaccines through cell culture technology. FCC technology has a number of advantages over traditional egg-based vaccines but the overall process is new, relative to the egg-based vaccine process, and still has a number of optimization and robustness challenges to overcome. This thesis focuses on the challenge of growing cells with a batch process to generate a high-density cell culture for infection with an influenza virus strain.

1.2 Approach

To generate the high-density cell culture necessary for infection, a simple design of experiment is used to screen important factors and then to optimize the batch process at a small scale within the laboratory. Discussions with a number of company personnel, along with a literature review of animal cell cultures, generated the list of factors to screen with the initial and then follow-up experiments. After initial experimental data was collected an analysis was performed to determine and model the effects and interactions of the various parameters. This model indicated the optimal conditions to maximize cell production and was used as a starting point for additional optimization at a larger laboratory scale.

Once this small scale data was collected and the most promising settings determined, the experiment was repeated at a larger laboratory scale so that the infection step could occur and data from the downstream processes could be collected. When the scale-up process failed to produce positive results the project focus shifted to an exploration of factors influencing scale-up. While the overall goal of the project was to improve the primary yield of the downstream

process, experimental results in the upstream process dictated that the research instead focus solely on batch cell growth.

1.3 Organization of the Thesis

Chapter One introduces the problem and briefly discusses the approach taken in this thesis to resolve the problem.

Chapter Two gives a background on Novartis, the V&D division, and the influenza vaccine industry.

Chapter Three describes the overall process of making flu vaccines using FCC technology and further details the process details related to this project.

Chapter Four describes the materials, equipment, and lab procedures used to conduct the experiments for this thesis.

Chapter Five covers the approach, data collected, and results of the experiments used to develop this thesis.

Chapter Six concludes the thesis and makes recommendations for future research to build on this thesis.

Chapter 2: Background

Chapter 2 gives a brief introduction to the internship host company Novartis and the influenza vaccine industry. This information provides some context before exploring the subject matter of the thesis.

2.1 Novartis and the Vaccines and Diagnostics Division

Novartis is a multinational pharmaceutical company that was created with the merger of Ciba-Geigy and Sandoz in 1996. Based in Basel Switzerland, Novartis is one of the largest health care companies ranking 3rd in sales of pharmaceutical companies in 2008 with $36.172 billion in sales[1]. Novartis divides their business into four sectors called Pharmaceuticals, Vaccines and Diagnostics, Consumer Health, and Sandoz which is their generics division[2]. Vaccines and Diagnostics, where this internship takes place, is the newest Novartis division and it was formed after Novartis acquired Chiron Corporation in 2006[3]. Novartis Vaccines and Diagnostics can be further broken down into Novartis Vaccines and Chiron blood testing which focuses on blood screening testing[4]. The Novartis Vaccines portfolio includes vaccinations against Influenza, Meningitis, Rabies, Japanese encephalitis, Tick-borne encephalitis, Haemophilus Influenzae type B (Hib), Polio, Diphtheria, Tetanus, and Pertussis (whooping cough)[5].

2.2 Influenza Vaccine General Information and Industry Background

An influenza, or flu, vaccine is used to deliver the main proteins of a particular flu virus strain into a person. Once a body receives these proteins it can create the antigens necessary to prevent the spread of that viral strain in the event that infection occurs. One of the main challenges of the influenza vaccine industry is the number of influenza strains in existence and how quickly these strains evolve.

While there are a great number of different influenza strains, the yearly flu vaccine is designed to protect against only three different subtypes of the virus. Typically, the vaccine protects against two strains from the Influenza A family and one from the Influenza B family. Influenza viral strains evolve so quickly that the vaccine from any given year rarely protects against the same strain as the previous year. The situation is further complicated by the division of the population into Northern and Southern hemispheres which have winter at different times

of the year and thus have different peak flu seasons and typically different dominant viral strains.

Strains in the yearly influenza vaccine are determined by the WHO as they collect data throughout the world and try to predict the strains that will be most prevalent in the upcoming flu season. The WHO works with a number of regional centers to collect influenza data and then works with vaccine manufacturers, who are constantly screening new strains with their manufacturing process, to select the strains for the upcoming flu season. Official strain selection information is released annually by the WHO for both the Northern and Southern hemisphere approximately 10 months before the flu season of that hemisphere begins. For the Northern Hemisphere, strain selection information is released between January and March and finished product must be available as soon as October. This short time period provides vaccine manufacturers with less than seven months to optimize their manufacturing process for the new strains and then to fully ramp up production. Many companies, Novartis included, will track and trend the popular virus strains along with the WHO, estimate the dominant strains before the WHO releases the final selection, and then begin production in an attempt to beat competition to market. This risk does not always pay off as product with incorrect strains must be discarded.

Chapter 2 introduced the company and industry to which the thesis pertains. The upcoming chapter will further explore the relevant process details pertaining to the thesis.

Chapter 3: Overview of Flu Cell Culture Process

Chapter 3 provides the background necessary to understand the overall FCC production process and some of the challenges that led to the use of batch experiments over fed-batch for high cell density infection.

3.1 Novartis Influenza Vaccine Production Process Overview

For Novartis to create a vaccine against a particular viral strain, that viral strain has to be grown, harvested, and then deactivated and purified so that the desired proteins from that virus can be injected into a patient. The two methods used to generate the large quantities of infected material are infection of embryonic chicken eggs and infection of animal cells that are grown in a bioreactor. While the two methods differ in their creation of infected materials, once this material is harvested the processes is similar.

Since the development of a working influenza vaccine in the 1940s, the traditional method of producing infected material for harvesting is with embryonic chicken eggs[6]. While egg-based production is a successful and proven process it has a number of challenges. One such challenge is the lack of flexibility in the sourcing of eggs and the supply chain for the process. Approximately one embryonic egg is required to produce one tri-strain vaccine and embryonic eggs require over six months of lead time[7]. This inflexibility prevents the process from accommodating deviations such as production failures, viral strain changes, or uncharacteristically high demand such as with a pandemic situation [8]. Embryonic eggs are also at risk of infection from viruses such as avian flu, which could decimate the raw material supply well after steps could be taken to replace the supply. Finally, persons allergic to eggs may have reactions to egg-based vaccines.

Challenges with the egg-based process led Novartis to research and develop a method of creating infected viral material using animal cell culture as the host. FCC research began in 1985 and reached small scale fermenter manufacturing runs by 1992[9]. Phase I and II clinical trial material was made in 2002 and phase III in 2004 and 2005[10]. The Novartis FCC vaccine has been released under the name Optaflu® and approval from the European Union was granted in 2007[11].

The cell culture process can be broken into upstream and downstream processes. The upstream process is diagramed in Figure 1: Downstream Process Overview and includes Steps

(1) seed-source cell expansion in multiple lab spinner flasks, (2) further cell expansion in consecutively larger bioreactors, (3) high-density cell expansion with a fed-batch process, (4) viral infection, (5) virus propagation as the virus spreads among the cells, (6) centrifugation to remove cells and debris, and (7) filtration to remove additional contaminants.

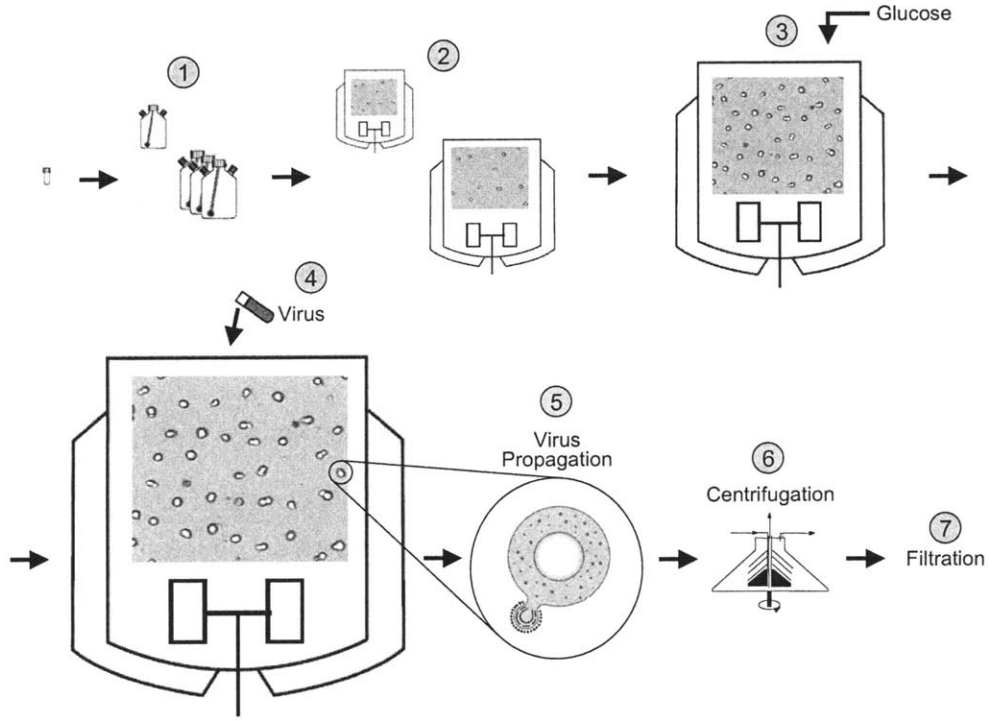


Figure 1: Downstream Process Overview

After filtration in the upstream process the product flows through a series of downstream processes which include virus inactivation, splitting and sub-unit extraction, and polishing purification. While the details of the downstream process are not relevant to this thesis the main function of the downstream process is to isolate the desired antigens and remove as many contaminants as possible so that the product is fit for injection in human patients.

3.2 Cell Growth Details

Overall, the laboratory processes used for experimenting are similar to the scaled up manufacturing process. For both processes the cells are expanded by adding a small solution containing cells to a growth media containing essential material for biological growth. Initially this cell expansion occurs in small incubated flasks, Step (1) of Figure 1: Downstream Process

Overview, with only temperature and headspace CO_2 controls. Next, the growth and then infection occurs in closed bioreactors with a number of additional process controls such as pO_2, glucose feed rate, and pH, Steps (2) - (5). In the laboratory these bioreactors are between one and eight liters and in the manufacturing process the bioreactors can be as large as 5,000 liters. All cell expansion steps except for the last stage typically increase the cell culture density by a factor of 10 at which point the solution is transferred to a larger vessel and diluted in growth media to the starting cell density for further expansion. The final high-density cell expansion, Step (3), typically increases the cell culture density by a factor of 30. The need to dilute the culture to the starting cell density at the end of each cell growth stage significantly increases the overall culture volume of the process, from one liter to 5,000 liters, and has a significant impact on the size of equipment in the manufacturing process.

3.3 Fed-Batch Process and Possible Batch Process

To achieve the high cell densities of the last expansion step, Step (3), the current process uses a fed-batch system. In a batch process all of the ingredients are added at the start of the reaction. Fed-batch implies that some ingredients are fed, as the reaction occurs, to what would otherwise be a batch process. For this process the only fed ingredient is glucose which serves as a food source for the cells. Glucose additional rates are determined by the number of cells currently in the bioreactor, so the glucose addition rate increases as cell growth occurs and varies depending on how efficiently the reaction is occurring.

Replacing the fed-batch step with a batch step is a simple change in this process. All of the glucose would be added to the growth media and cell solution at the beginning of the final cell expansion step. After that, the cell growth would continue until the high-density concentration level is achieved. A batch process has the benefit of simplicity compared to a fed-batch process. In the fed-batch process, glucose must be adjusted daily depending on the current cell concentration. This action introduces variability in the process and makes the manufacturing process difficult to schedule and manage. A batch process requires no daily measurement of cell count and no adjustment of the glucose feed rate and thus has a lower chance for variability compared to the fed-batch system.

The current manufacturing process is verified up to a cell density level that can be consistently reached with a fed-batch process. If another process for growing cells, such as a

batch system, can consistently reach a higher cell density it would allow for a larger number of infected cells in the final bioreactor with the same dilution ratio. As will be detailed in the High-Density Cell Infection section, a higher cell density at infection may lead to an improvement in overall yield.

3.4 High-Density Infection

Through past studies performed by Novartis it is known that final product yield increases as infection cell density increases, as seen in Figure 2: Infection Cell Density versus SRD Yield.

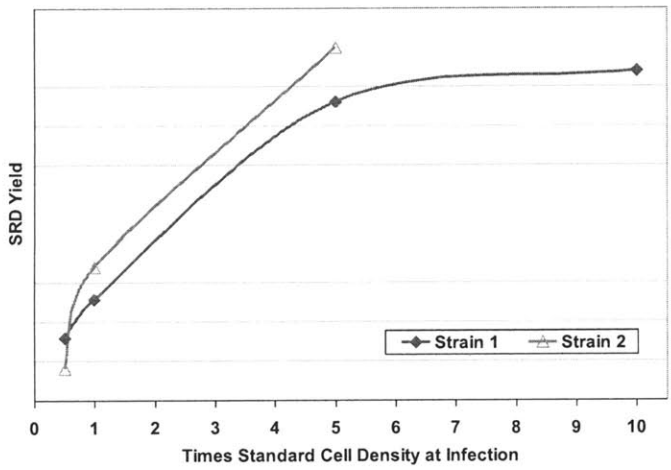


Figure 2: Infection Cell Density versus SRD Yield[12]

For the research referenced in Figure 2: Infection Cell Density versus SRD Yield, cultures with a higher cell density than the standard cell density were generated through a perfusion process. A perfusion process involves feeding fresh media into a culture while withdrawing an equal volume of spent media. While this method was able to generate cultures that are ten times as dense as the standard culture with a final yield that was three times greater, the economics of continuously feeding fresh media did not make this a feasible production process.

In the standard fed-batch process, the contents of the cell growth bioreactor are transferred to a larger bioreactor containing a different media than the one used during cell growth. As shown in Figure 1: Downstream Process Overview Step (4), infection occurs in a bioreactor that is larger than the bioreactor used to grow the cells and a different media is used for infection than the media used for cell growth. It should be noted that the perfusion process used for

Figure 2: Infection Cell Density versus SRD Yield, which exchanges old media for new media, could have removed contaminants and would therefore be misleading on the effectiveness of high density infection.

This chapter has outlined the overall FCC production process and then given detail relevant to this thesis for cell growth through a batch or a fed-batch process. The chapter concludes with a discussion of the historical data that led to this thesis and why it is believed that batch system will be successful. As stated in this chapter, the purpose of this thesis is to test if batch grown cells follow the trend in Figure 2: Infection Cell Density versus SRD Yield where a higher cell density leads to a higher overall process yield. In the next chapter we will review the material, equipment, and methods used in this thesis before explaining the approach taken for this project in the following chapter.

Chapter 4: Material, Equipment, and Methods

This chapter covers the different laboratory materials, equipment, and methods used to conduct the experiments outlined in this thesis.

4.1 Material

4.1.1 Cells

The Novartis FCC process has been developed with MDCK epithelial cells as the host cells for infection. The MDCK cell line was initiated in 1958 by S. H. Madin and N. B. Darby from the kidney tissue of an adult female cocker spaniel[13]. The MDCK line is susceptible to a number of viruses besides the influenza virus which makes it a popular tool for study[14]. Novartis maintains their own particular MDCK cell line called MDCK 33016 in an internal working cell bank to preserve the integrity of the cell line.

4.1.2 Chemical Defined Media

During the various cell growth stages, a small quantity of cell culture is added to a large quantity of media at a set ratio to meet a targeted cell density. This media provides the ingredients and the overall dilution necessary for cell growth.

4.1.3 Other Cell Culture Growth Additives

Concentrated solutions of Amino Acids, Vitamins, Trace Metals, Glucose, etc. were used to vary individual parameters for designed experiments. A list of the different chemicals used during any experiment and any pertinent information about said chemicals can be found in Appendix 1: Raw Material Information.

4.2 Equipment

4.2.1 Bioreactor

For these experiments the bioreactors used were one liter glass vessels with a culture volume of 1.1 liters. The controls for the bioreactor were managed with a BIOSTAT Q

system that controls headspace gas flow and content, pO_2, pH, temperature, and stir speed. Temperature is maintained by pumping hot or cold water through the insulation system surrounding the bioreactor and stir speed is controlled through a top-loaded variable speed motor that rotates a central shaft. The stirring device is a single pitched blade impeller with down-pumping agitation. To control both pH and pO_2 the system manages a constant flow of headspace gas at a controlled ratio of air, CO_2, and nitrogen. Further control of pO_2 is through a sparger that delivers oxygen gas to the bottom of the vessel and further pH control is by the addition of a sodium hydroxide solution to the culture.

4.2.2 Glass Vessel

The 100 milliliter, one liter, and two liter vessel are sterilized glass vessels of cylindrical shape with sealable lids used to simulate a bioreactor. To allow for air exchange during cell growth, the caps are loosened but not removed from the vessel once placed in the incubator according to currently used lab policy.

4.2.3 Incubator

The incubator used for the shaker flasks experiments was a Heraeus 6000. The incubator is certified for and was maintained at the targeted temperature ± 1°C and at the targeted % CO_2 ± 1% CO_2.

4.2.4 Shaker Flask

Disposable plastic shaker flasks are used as a small-scale simulation of bioreactors. The polycarbonate erlenmeyer/shaker culture flasks were 250 mL flasks equipped with a DuoCAP which allows for sterile air exchange. A glass shaker flask with a sealable plastic lid was also used to explore the impact of vessel material. To allow for air exchange during cell growth, the caps are loosened but not removed from the vessel once placed in the incubator according to currently used lab policy

4.2.5 Shaker Plate

A Sartorius Certomat MOII shaker plate inside the incubator provided rotational agitation for the shaker flasks. While the shaker plate is capable of various speeds the plate agitation remained constant for all experiments.

4.3 Methods

A list of the standard information collected for the experiments conducted in this thesis can be found in

Appendix 2: Experiment Test Information. Further pertinent information about the methods used is listed below.

4.3.1 Cell Count and Viability

To determine the cell density of viable cells in a solution the Beckman Coulter Vi-cell XR viability analyzer was used. This vi-cell measuring device has been verified within the Novartis laboratory and is their standard method of cell density measurement.

4.3.2 Nova Analyzer

To measure the concentration of glucose, glutamine, glutamic acid, lactate, ammonia ion, and the osmolality a Nova Biomedical 100 Plus Bioprofile Analyzer was used.

4.3.3 PHOX Analyzer

To measure the dissolved O_2 and CO_2 a Nova Biomedical Bioprofile PHOX was used.

4.3.4 Sterile Handling and Sampling Techniques

For initial cell expansion and spinner flask work cells, and other components, were transferred within a laminar flow hood using sterile lab equipment. Daily samples were taken with a sterile pipette within the same laminar flow hood.

Bioreactors were assembled, sealed, and then autoclaved to ensure sterilization. Additions and samples were made through sterile valve connections that were attached to the vessel before autoclaving. Headspace pressure was used to draw out the sample and a series of clamps were used to ensure that no contamination occurred.

Now that the key material, equipment, and methods have been detailed the next chapter will discuss how these things were used to conduct the experiments for this project.

Chapter 5: Strategy to Evaluate High Cell Density Infection of Batch Grown Cells

In an attempt to improve the overall process yield, the goal of this evaluation is to determine if a batch generated cell culture can be infected at a higher cell density than the current fed-batch process allows and if that higher cell density at infection leads to higher yields. The following chapter will discuss the approach used to determine the feasibility of batch cell growth for high-density infection, present the results from the experiments conducted, and then analyze and summarize the results.

5.1 Approach

The approach for this project is outlined in the graphic below and can be broken into the two key components of high-density batch cell growth followed by high-density infection. The first step is to determine the feasibility of generating a high density cell culture from a batch process at a small shaker flask scale and then explore the addition of other supplements. Following small scale optimization is bioreactor replication which then allows for the final phase of high-density infection. When the one liter bioreactor experiments failed to produce positive results, the project focus shifted to scale-up exploration and eventually small scale reproducibility.

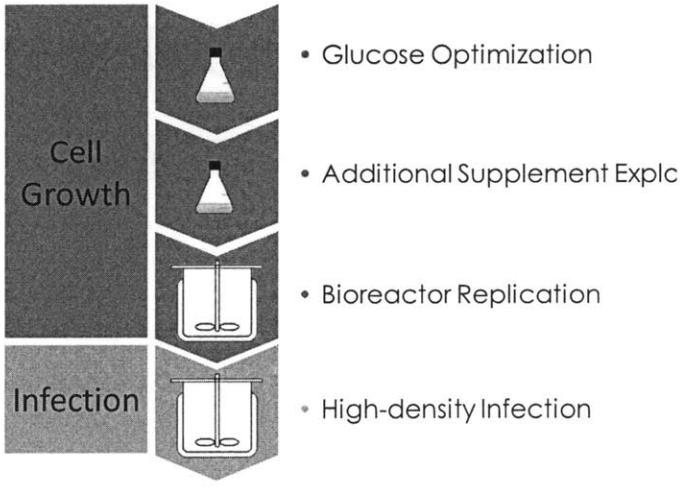


Figure 3: Approach

5.1.1 Experiment 1: Glucose Screening in Shaker Flasks

If glucose feeding is required for high density cell growth in a standard fed-batch process, the batch process will require a substantial amount of initial glucose. For both equipment scheduling and economic reasons the initial glucose screen took place in 250 mL shaker flasks. Within each flask was added a dilution of cells in the current cell growth media at the standard starting cell density with a total culture volume of approximately 100 mL. Next, concentrated glucose was added to adjust the initial glucose level between the standard fed-batch level and eight times the standard level as seen Table 2: Experiment 1 - Design.

Table 2: Experiment 1 - Design

Flask Number	Initial Glucose Concentration (% of Standard)
1	0
2	200
3	300
4	500
5	700
6	800

These samples were then cultivated by agitating the flasks on a shaker plate placed inside an incubator that controls temperature and headspace CO_2. Peak cell concentration was achieved after more than seven days of cultivation. A daily sample was pulled from each flask to measure the parameters listed in

Appendix 2: Experiment Test Information.

For cell growth experiments, viable-cell concentration was used as the primary response parameter with the assumption that more cells available for infection will lead to a higher final yield. While this parameter may not correlate directly to final yield, no better measurement is available at the smaller laboratory scale because these samples are not large enough to infect and process through downstream.

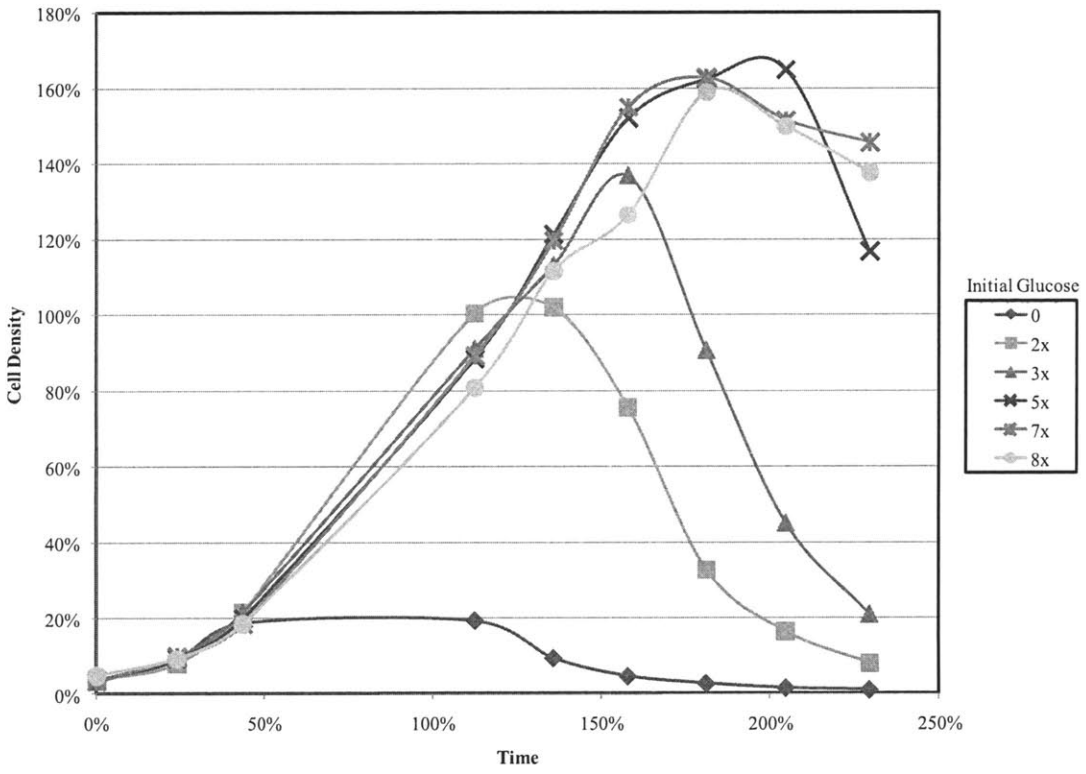


Figure 4: Experiment 1 Results - Cell Density vs. Time

As seen in Figure 4: Experiment 1 Results - Cell Density vs. Time, the cell concentration of the batch processes with additional glucose far exceeded the typical fed-batch cell concentration of 100%. By plotting the peak cell concentration achieved for each experiment, as seen in Figure 5: Experiment 1 Results - Peak Cell Density vs. Initial Glucose, we can see that the optimal initial glucose concentration is slightly more than five times greater than the original initial glucose concentration. Going forward, all experiments

will be centered around an initial glucose concentration that is five times greater than the standard initial glucose concentration.

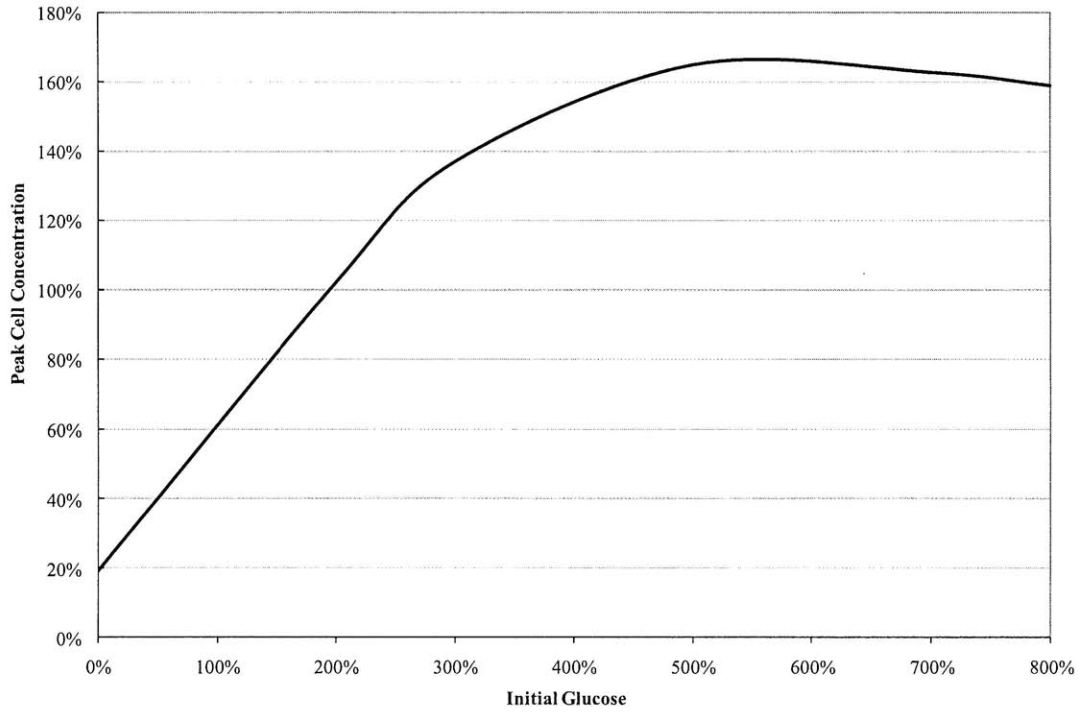


Figure 5: Experiment 1 Results - Peak Cell Density vs. Initial Glucose

5.1.2 Experiment 2: Media Supplement Screening in Shaker Flasks

Using the optimal initial glucose concentration from the first experiment results as a starting point, additional factors were then screened in similar shaker flask experiments. The purpose of this set of experiments was to identify key parameters that influence cell growth. Discussions with a number of company personnel, along with a literature review of animal cell cultures, determined that the parameters to screen first were amino acids, vitamins, and trace metals. A full factorial DOE with three centerpoints was then used to screen these three factors along with a narrow range of glucose addition as the fourth factor. The experimental layout can be seen in Table 3: Experiment 2 – Glucose, Trace Metals, Amino Acids, and Vitamins.

Table 3: Experiment 2 – Glucose, Trace Metals, Amino Acids, and Vitamins

Run Order	Glucose	Trace Metals	Amino Acids	Vitamins
1	+	+	–	+
2	+	+	+	–
3	–	+	+	–
4	+	–	–	–
5	–	–	–	–
6	0	0	0	0
7	0	0	0	0
8	+	+	+	+
9	+	+	–	–
10	+	–	+	+
11	+	–	–	+
12	+	–	+	–
13	–	–	+	+
14	–	–	+	–
15	–	–	–	+
16	–	+	–	+
17	0	0	0	0
18	–	+	+	+
19	–	+	–	–

For this experiment, the standard culture media was used and various additives were used to change the composition of this media. Another option would have been to use a different culture media with the desired composition. The decision to use the current culture media as a base and add various elements individually, rather than to use a new culture media, was driven from a validation perspective. The change management requirements to make modifications to the current media formulation is significantly less work than the work required to implement a new media culture.

A full factorial DOE was used during this exploratory DOE stage because shaker flasks are more economical than bioreactor experiments making them a more cost effective way to identify interactions between parameters. As with Experiment 1, within each flask was a total culture volume of approximately 100 mL consisting of the current growth media, cells, and additional components according to the DOE criteria. For Trace Metals, Amino Acids, and Vitamins, – signifies that no additional amount of that component is added. The + signifies that concentrations of that parameter were increased between 20% and 50% through the use of concentrated solutions. 0 signifies a parameter increase at the midpoint

between – and +. For glucose, 0 signifies the optimal concentration determined from the previous experiment, a 500% of the growth media's initial glucose concentration, and the – and + symbol signify a 10% concentration decrease and increase from that value, respectively.

As seen in Figure 6: Experiment 2 Results - Cell Density vs. Time, the average results from the three midpoint runs from Experiment 2: Media Supplement Screening in Shaker Flasks were nearly 2.5 times better than the traditional fed-batch process.

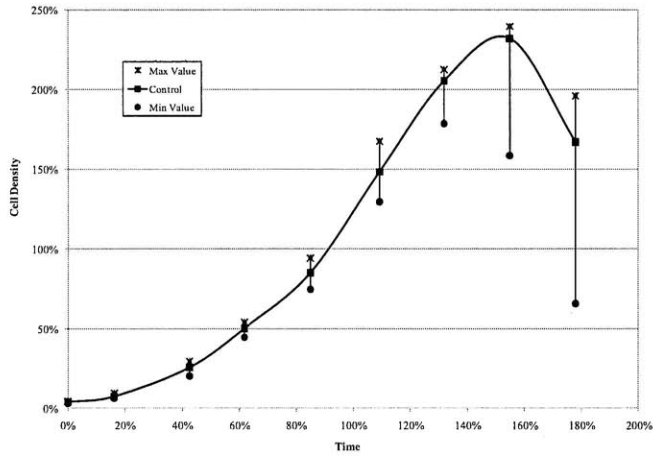


Figure 6: Experiment 2 Results - Cell Density vs. Time

A linear regression will be performed to analyze a number of experiments in this thesis. Details of the analysis for Experiment 2: Media Supplement Screening in Shaker Flasks, including the steps to validate the regression, will be reviewed here. All future linear regression results, following this same model confirmation steps, will be listed in the appendix and referenced throughout the thesis.

For Experiment 2: Media Supplement Screening in Shaker Flasks, cell density is the response parameter and initial glucose concentration, amino acid content, vitamin content, and trace metal content are the independent variables. After generating the regression model using JMP 8.0 and eliminating parameters that do not significantly impact the model, the final regression results can be seen in Appendix 3: Experiment 2 – Statistical Analysis of Glucose, Trace Metals, Amino Acids, and Vitamins. The first model output to verify is the F test to confirm that the response parameter significantly depends on the model parameters.

With an F test value of 7.18 with a p value of less than 0.05 we may conclude that the response parameter does depend on the model parameters.

The next model confirmation is the R^2 value which indicates how well the model fits the data. An R^2 value of 0.9349 indicates that we have captured most of the variance with the model and the adjusted R^2 of 0.8047 indicates that we have not overfit the model.

Next, a plot of the residuals versus prediction, row, and each of the effects all show the residuals to be randomized and not systematic. Furthermore, a look at the normal plot of the residuals indicates that there are no residual outliers because all points are contained within the 95% confidence band and seem to follow a random pattern along the line.

Having confirmed that our model is sound, a look at the t-test indicates which of the modeled parameters most influence the response parameter. The t-test values indicate that the addition of a trace metal supplement hinders cell growth and that glucose is the only other significant factor influencing cell growth and all other main effects and interactions are insignificant.

As seen in Figure 7: Experiment 2 Results - Glucose and Cell Density vs. Tim, glucose is still available in the culture when cell growth slows and eventually stops. While the regression model undoubtedly proves that glucose is a dominating factor for cell growth, it fails to identify the limiting or inhibiting parameter preventing further cell growth.

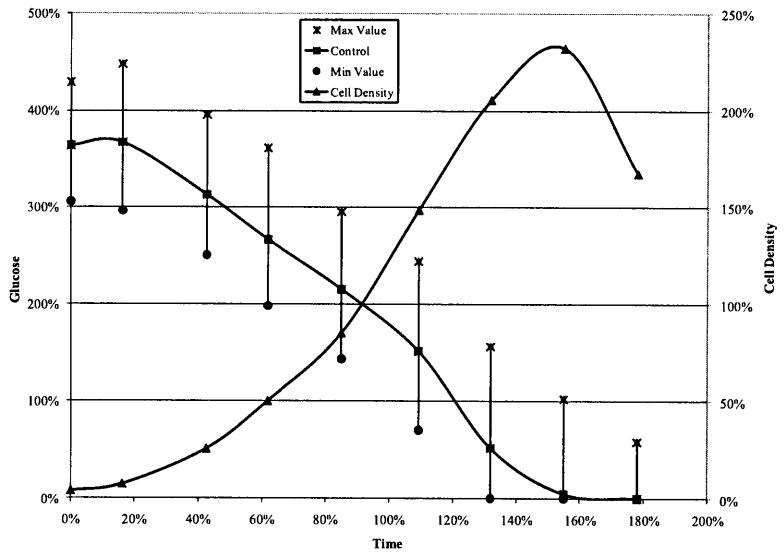


Figure 7: Experiment 2 Results - Glucose and Cell Density vs. Time

Additional trends of measurements taken during this experiment are available in Appendix 4: Experiment 2 - Cell Density vs. Time through

Appendix 11: Experiment 2 – Glutamic Acid vs. Time. The final conclusion to draw from this experiment is that batch cell growth is achievable with the standard cell growth media and the addition of trace metals, amino acids, and vitamins did not significantly improve this growth. As was show in Experiment 1: Glucose Screening in Shaker Flasks, and confirmed in this experiment, the key parameter in batch cell growth is the initial glucose concentration. The next step is the replication of these results in a one liter bioreactor so that infection experimentation can occur.

5.1.3 Experiment 3: Bioreactor Replication

Shaker flasks, while cost effective and easy to use, have a number of differences from the bioreactors used by the manufacturing process. Experiment 3 aims to replicate, in a one liter bioreactor, the shaker flask results of Experiment 1: Glucose Screening in Shaker Flasks and Experiment 2: Media Supplement Screening in Shaker Flasks. Bioreactors, compared to shaker flasks, allow for additional process controls including the control of pO_2, pH, temperature, and stir speed. Bioreactors also use a different CO_2 scheme from shaker flasks.

pH control, pO_2 setting, and temperature setting were chose for the initial DOE and a half factorial was used to allow for the maximum amount of screening of the main effects with the minimum amount of runs as seen in Table 4: Experiment 3 – pH control, pO_2, and Temperature.

Table 4: Experiment 3 – pH control, pO_2, and Temperature

Run Order	pH Control	pO_2 Set Point	Temperature Set Point
1	Control	+	–
2	Control	–	+
3	No Control	–	–
4	Control	0	0
5	No Control	+	+
6	Control	–	+
7	No Control	–	–
8	No Control	+	+
9	Control	+	–

Control of pH was one of the tests selected because of the difference between how CO_2 is controlled with shaker flasks and bioreactors. The bioreactor system controls pH by introducing CO_2 into the overlay gas when the pH is too high and base, 0.5 M sodium hydroxide, when the pH is too low. In a shaker flask, the percent of CO_2 in the overlay gas is held at a constant 5% and otherwise pH is not controlled. For this DOE, pH was set for runs labeled Control and not set, or no pH controls were used, for runs labeled No Control.

pO_2 was selected because previous experiments with these shaker flasks suggested that the agitation required for good cell mixture results in a pO_2 that is significantly higher than the typical setting for a bioreactor[15]. The pO_2 setpoint was varied from 70% of the normal value to 130% of normal, represented by – and +, with a midpoint of 100%, represented by 0.

Temperature was selected because in past bioreactor experiments temperature is often a key parameter[16]. Temperature was varied from 0°C to 1°C, represented by – and +, with a midpoint of 0.5°C, represented by 0.

As seen in Figure 8: Experiment 3 – Cell Density vs. Time, the results from the bioreactor replication show that cell growth in the bioreactors was significantly lower than cell growth in shaker flasks. The graph also clearly shows that turning off the pH controller

negatively impacts cell growth, which is also confirmed by the regression analysis seen in Appendix 12: Experiment 3 – Statistical Analysis of pH, pO2, Stir Speed, and Temperature.

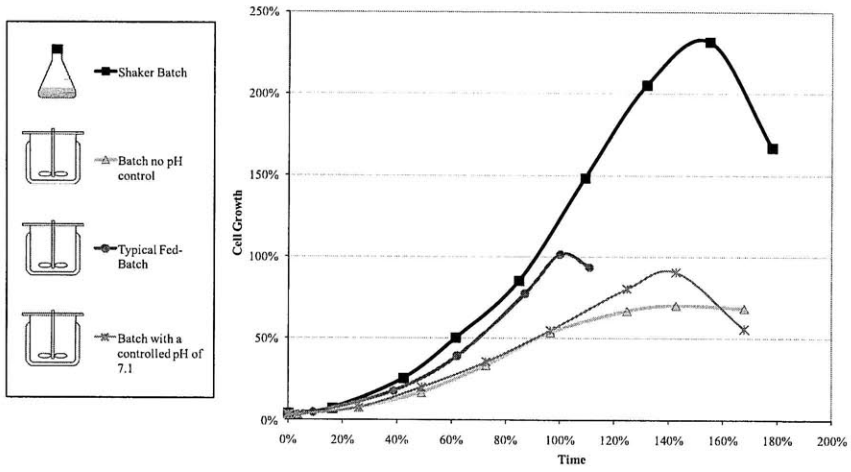


Figure 8: Experiment 3 – Cell Density vs. Time

The failure of the bioreactor to reproduce the positive shaker flask results created the need for another bioreactor DOE. As previously detailed, the CO_2 control scheme for a bioreactor is different from the control scheme of a shaker flask. Equipment availability limited this special testing to one bioreactor, but an attempt was made to more realistically replicate the CO_2 control scheme of a shaker flask in a bioreactor. To do this, a pump capable of delivering a constant flow of 5% CO_2 was attached to the bioreactor's overlay gas. Stir speed was also increased to 190% of the normal rotations per minute in this one bioreactor to try to simulate the excessive agitation of a shaker flask. In addition to this one bioreactor, a typical DOE was setup to test a lower pH value, higher stir speeds, and a wider variety in temperature. The design can be seen in Table 5: Experiment 3 – Stir Speed, Temperature, and CO2 Control. Stir speed varies from 100% of the normal rotations per minute to 190%, designated – and +, and temperature varies from -4°C to 0°C, designated – and +.

Table 5: Experiment 3 – Stir Speed, Temperature, and CO_2 Control

Run Order	Stir Speed	Temperature	CO_2 control	pH Set Point
1	-	-	Normal	-0.1
2	+	+	Normal	-0.1

3	+	-	Normal	-0.1
4	-	-	Normal	-0.1
5	+	+	5% Constant	None

Like the first bioreactor replication DOE, as can be seen in Figure 9: Experiment 3 – Cell Density vs. Time, none of the bioreactors matched the results achieved with shaker flasks in Experiment 2: Media Supplement Screening in Shaker Flasks.

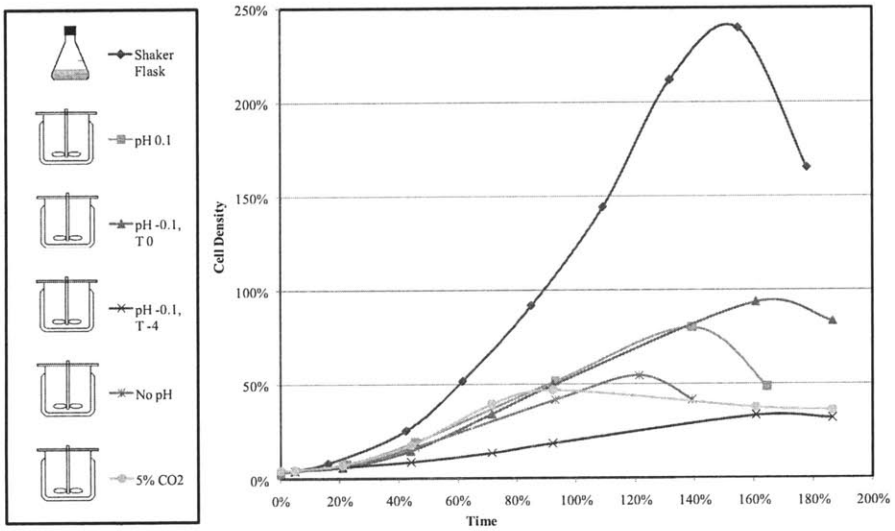


Figure 9: Experiment 3 – Cell Density vs. Time

A regression analysis of all of the experiments with a controlled pH set point, as seen in Appendix 13: Experiment 3 – Bioreactors with pH Control - Statistical Analysis of Temperature, pH, pO_2, and Stir Speed, demonstrates that temperature, pH, pO_2, and initial glucose concentration do have a significant effect on batch cell growth. However, the effect of these parameters on cell density is small relative to the difference between the cell densities achieved in Experiment 2: Media Supplement Screening in Shaker Flasks and Experiment 3: Bioreactor Replication.

Graphs of all key tests for this experiment can be seen in Appendix 14: Experiment 3 - Cell Density vs. Time through Appendix 21: Experiment 3 - Glutamic Acid vs. Time and observations from these charts are included in Appendix 24: Comparison of Shaker Flasks from Experiment 2 and Bioreactors from Experiment 3. While the table discusses all of the major parameters, the key takeaways are that glucose consumption was higher in the

bioreactors despite producing fewer cells than the shaker flasks, lactate and ammonium production are tied to the bioreactors settings and at higher levels may be inhibiting growth, and media age may be influencing cell growth.

To explore potential scale-up inhibiting parameters, Appendix 25: Comparison of Shaker Flask and Bioreactor Systems offers a comparison of the shaker flask system with the bioreactor system. Of the system parameters compared, the most promising parameters to explore are the ones influencing CO_2. The next step is to run bioreactors and shaker flasks, filled with the same culture source, in parallel while measuring CO_2 to try to understand the difference between the two systems.

5.1.4 Experiment 4: Parallel Experiments with Shaker Flasks and Bioreactors

While the past experiments have shown that cell growth in Experiment 3: Bioreactor Replication is significantly less than cell growth in Experiment 2: Media Supplement Screening in Shaker Flasks, the reason for this disparity is still not yet clear but the most promising parameters to test are those that influence dissolved CO_2. The next experiment was designed to test shaker flasks and bioreactors in parallel by filling both vessels with the same pre-culture. The bioreactor setups can be seen in Table 6: Experiment 4 - Bioreactor Setup and the shaker flask setups can be seen in Table 7: Experiment 4 - Shaker Flask Setup.

Table 6: Experiment 4 - Bioreactor Setup

Stir Speed	Temperature	pO_2	CO_2 Control	pH Set Point	Surface Area to Liquid Volume Ratio
100%	0	100%	Normal	-0.1	0.57
100%	0	100%	Normal	-0.1	0.40

Table 7: Experiment 4 - Shaker Flask Setup

Test	Vessel	Vessel Material	Surface Area to Liquid Volume Ratio	Time Pulled from Bioreactor (%)
Pulled from bioreactor	Shaker Flask	Polycarbonate	0.50	0%
Pulled from	Shaker Flask	Polycarbonate	0.50	0%

bioreactor				
Pulled from bioreactor	Shaker Flask	Polycarbonate	0.50	6%
Pulled from bioreactor	Shaker Flask	Polycarbonate	0.50	12%
Pulled from bioreactor	Shaker Flask	Polycarbonate	0.50	23%
Pulled from bioreactor	Shaker Flask	Polycarbonate	0.50	46%
Control	Shaker Flask	Polycarbonate	0.50	-
Control	Shaker Flask	Polycarbonate	0.50	-
Control	Shaker Flask	Polycarbonate	0.50	-
Control	Shaker Flask	Polycarbonate	0.50	-
Material	Shaker Flask	Glass	0.50	-
Shape and Surface/Liquid Volume Ratio	1 L Bottle	Glass	0.57	-
Shape and Surface/Liquid Volume Ratio	2 L Bottle	Glass	0.98	-
Shape and Surface/Liquid Volume Ratio	100 mL Bottle	Glass	0.87	-
Agitation Method	Internal Stir Bottle	Glass	0.51	-

As seen in the experimental design, also explored are different surface area to liquid volume ratios in the bioreactors and vessel shape, material, and surface area to liquid volume in the shaker flasks. Included is also a set of shaker flask experiments that contain culture that was removed from batch bioreactors at different points during the reaction. The purpose of this is to determine at what point in time during the cell growth in the bioreactor are the contents of the culture exhausted or altered into a culture that is not longer able to achieve the high density growth achieved in Experiment 2: Media Supplement Screening in Shaker Flasks. As seen in Figure 9: Experiment 3 – Cell Density vs. Time, there is a clear difference in cell growth between the shaker flask and bioreactor visible before time reaches 40%.

One final component incorporated into this experiment is the measuring of dissolved CO_2 and O_2 in both the shaker flasks and bioreactors. Measuring CO_2 is not a typical

procedure at the lab where these experiments were conducted and a measuring device was not available before this experiment.

Like the previous bioreactor experiments, the bioreactors in this experiment, as seen in Figure 10: Experiment 4 – Cell Density vs. Time, failed to reach the same cell growth that was achieved in Experiment 2: Media Supplement Screening in Shaker Flasks. Surprisingly, the shaker flask controls also failed to repeat the cell growth from Experiment 2: Media Supplement Screening in Shaker Flasks.

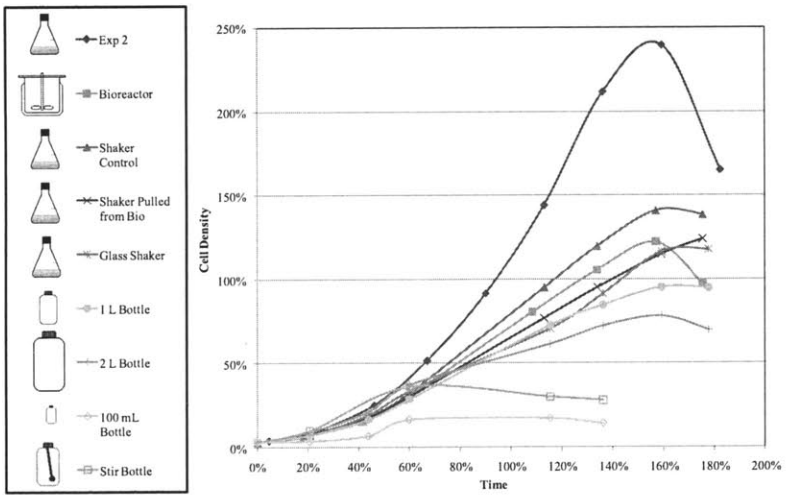


Figure 10: Experiment 4 – Cell Density vs. Time

A linear regression of the experiment, seen in Appendix 26: Experiment 4 - Statistical Analysis of Vessel, Material, and Time Pulled from Bioreactor, shows the impact of the parameters tested in this experiment, but with the shaker flask controls failing to reach a high cell density, the more obvious learning from this experiment is that the results achieved in Experiment 2: Media Supplement Screening in Shaker Flasks are not consistent and an unknown parameter is influencing cell growth.

Graphs of all key tests for this experiment can be seen in Appendix 27: Experiment 4 – Cell Density vs. Time through Appendix 35: Experiment 4 – pCO_2 vs. Time and observations from these charts are included in Appendix 36: Comparison of Shaker Flasks from Experiment 2 and Experiment 4. Of the parameters compared, the most promising parameter to explore is the influence of media age and the influence of the starting concentration of glutamine and ammonia as those concentrations are influenced by media

age. The next step is to run shaker flask experiments with media of a different age as well as with different initial concentrations of ammonium, glutamine, and lactate.

5.1.5 Experiment 5: Media Age, Lactate, Ammonium, and Glutamine Exploration

Media age is a parameter that stands out when analyzing why the control samples from Experiment 4: Parallel Experiments with Shaker Flasks and Bioreactor do not repeat the results seem in Experiment 2: Media Supplement Screening in Shaker Flasks. Cold storage of media extends the useful life of media and while all media used for these experiments were within the useful life of the product, Appendix 30: Experiment 4 – Ammonium vs. Time and Appendix 33: Experiment 4 – Glutamine vs. Time, shows the difference between the starting level of glutamine and ammonium due to the degradation of glutamine over three months. While ammonium and lactate have been to inhibit MDCK cell growth the ammonium and lactate levels cited in the study are significantly higher than the levels seen in the shaker flask and bioreactor experiments of this study[17]. Nevertheless, the influence of media age, glutamine, lactate, and ammonium on cell growth will be explored and the experimental setup can be seen in Table 8: Experiment 5 - Media Age, Lactate, Ammonium, and Glutamine.

Table 8: Experiment 5 - Media Age, Lactate, Ammonium, and Glutamine

Order	Media Age (days)	Lot #	Pre-charge Media	Initial Lactate	Initial Ammonium	Target Initial Glutamine
1	221	9SP106I	No	1	1	-
2	221	9SP106I	No	2	1	-
3	221	9SP106I	No	3	1	-
4	221	9SP106I	No	1	2	-
5	221	9SP106I	No	2	2	-
6	221	9SP106I	No	3	2	-
7	221	9SP106I	No	1	3	-
8	221	9SP106I	No	2	3	-
9	221	9SP106I	No	3	3	-
10	144	9SP140C	No	-	1	-
11	144	9SP140C	No	1	-	-
12	144	9SP140C	No	1	1	-

13	144	9SP140C	No	-	-	+
14	144	9SP140C	No	1	1	+
15	144	9SP140C	No	-	-	-
16	144	9SP140C	No	-	-	-
17	221	9SP106I	No	-	-	-
18	144	9SP140C	No	-	-	-
19	221	9SP106I	Yes	-	-	-
20	144	9SP140C	Yes	-	-	-

As seen in the experimental design, the normal media, lot 9SP106I, is at the time of this experiment 221 days old and the newer media, lot 9SP140C, is 144 days old. Samples 1-9 vary the initial ammonium and lactate levels, with 1, 2, and 3 representing 100%, 225%, and 350% for lactate and 1, 2, and 3, representing 200%, 350%, and 450% for ammonium. Samples marked with – did not have their ammonium or lactate adjusted. Samples 10-14 attempt to simulate starting levels for ammonium, lactate, and glutamine of the older media with the newer media. For glutamine, + indicates an initial glutamine level of 133% and – indicates no adjustment made to glutamine. Samples 19 and 20 test the influence of pre-charging the media the day before the cells are charged.

Graphs for this experiment can be seen in Appendix 39: Experiment 5 – Cell Density vs. Time through Appendix 46: Experiment 5 – Glutamic Acid vs. Time and observations from these charts are included in Appendix 47: Discussion of Results from Experiment 5. Figure 11: Experiment 5 – Cell Density vs. Time shows that once again the control shaker flasks failed to achieve the results of Experiment 2: Media Supplement Screening in Shaker Flasks.

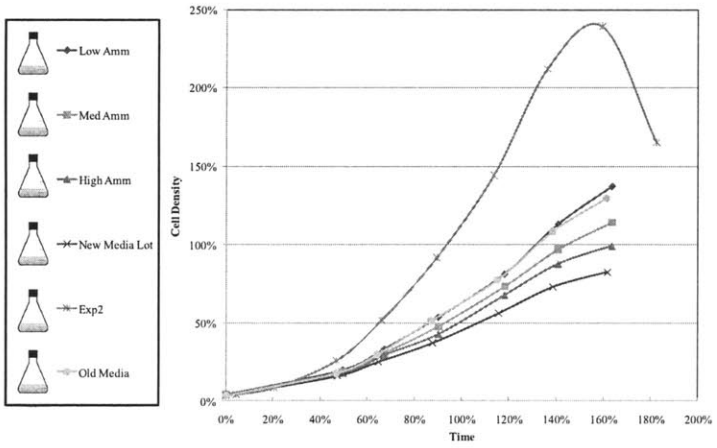


Figure 11: Experiment 5 – Cell Density vs. Time

We also see that the control experiments from both media lots further demonstrates the variability of batch cell growth as the new media lot does not even reach a cell density of 100% while the older media reaches about 135% for no explainable reason. Also seen is the clear influence that initial ammonium has on cell growth which is further confirmed by the linear regression seen in Appendix 38: Experiment 5 - Statistical Analysis of Glutamine, Lactate, Ammonium, and Media Age.

While there are further avenues to explore to try to account for the difference seen between Experiment 2: Media Supplement Screening in Shaker Flasks and all following experiments, time constraints forced this project to conclude.

5.2 Summary of Results

Experiment 1: Glucose Screening in Shaker Flasks determined the optimal starting glucose range using shaker flasks and was followed by Experiment 2: Media Supplement Screening in Shaker Flasks, another shaker flask experiment, which ruled out other supplements improving batch cell growth. Through the first two experiments the results achieved were excellent. Next, Experiment 3: Bioreactor Replication attempted to scale up the batch process to a one liter bioreactor while testing the influence of a number of adjustable parameters. When no ideal conditions were found for a bioreactor to replicate the initial results, Experiment 4: Parallel Experiments with Shaker Flasks and Bioreactors attempted to run bioreactors and shaker flasks in parallel to eliminate as many differentiating parameters as possible.

Surprisingly, the shaker flasks controls from Experiment 4 failed to reproduce the results from Experiment 2: Media Supplement Screening in Shaker Flasks. After testing the most prominent difference between Experiment 2: Media Supplement Screening in Shaker Flasks and Experiment 4: Parallel Experiments with Shaker Flasks and Bioreactors, media age and associated parameters, with shaker flasks and finding no conclusive reason for the variability the project was concluded due to time constraints.

Conclusion

Despite the negative results of this thesis, batch cell growth of MDCK still has potential as a process going forward. While the results were not consistent, the positive results observed from the experiment deem further exploration. Past experimental data has shown that a higher cell density at infection results in a higher final yield, and the batch process has shown its potential to reach higher cell densities, though inconsistently. It is important to note that the cause of the variation could be something that has always existed in the fed-batch process. Nearly every batch experiment achieved the cell density target of a fed-batch process. Variability occurred at cell densities above the typical operating range of a fed-batch process, so all aspects of the process should be considered as potential sources of variability. Further research is needed to identify what is causing the variability in results but given the potential improvement to the manufacturing process, this exploration is justifiable.

Appendix

Appendix 1: Raw Material Information

Product	Supplier	Lot	Other Remarks
R7131 Amino Acids Solution 50X	Sigma	049K2411	
33016 MDCK CDM	Lonza	9SP106I	Manufactured on 4/27/09
33016 MDCK CDM	Lonza	9SP140C	Manufactured on 7/13/09
Glucose Solution	Internal	SOP105098_0100	400 g/L
L-Glutamine	Sigma	45K23141	
L4263 Sodium DL-Lactate	Sigma	116K53064	Approx. 98%, 60% (w/w)
Trace Element Solution 50X	Gibco	1175172	
R7256 Vitamin Solution 100X	Sigma	019K2380	
A7219 L-Aspartic acid	Sigma	079K0104	
G8415 L-Glutamic acid	Sigma	128K0018	
V0513 L-Valine	Sigma	078K0039	
A8094 L-Arginine	Sigma	078K0178	
69775 Lactic acid	Sigma	1433079 41209P09	
P2126 L-Phenylalanine	Sigma	000144-3420	
22, 122-8 Ammonium hydroxide solution	Sigma	S87552-259	

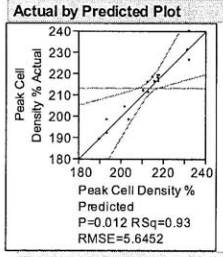
Appendix 2: Experiment Test Information

Method	Test	Actual Units	Basis for Thesis Units
Vi-Cell Analyzer	Cell Viability	% Live Cells	-
Vi-Cell Analyzer	Total Cell Concentration	cells/mL	Percentage of fed-batch target peak cell density
Vi-Cell Analyzer	Viable Cell Concentration	cells/mL	Percentage of fed-batch target peak cell density
Vi-Cell Analyzer	Cell Diameter	microns	-
Off-line pH probe	Off-line pH	$-\log_{10}[H^+]$	Difference from standard pH
NOVA Analyzer	Glutamine	g/L	Percentage of initial fed-batch value
NOVA Analyzer	Glutamic Acid	g/L	Percentage of initial fed-batch value
NOVA Analyzer	Glucose	g/L	Percentage of initial fed-batch value
NOVA Analyzer	Lactate	g/L	Percentage of initial fed-batch value
NOVA Analyzer	Ammonium	g/L	Percentage of initial fed-batch value
NOVA Analyzer	Osmolality	mOsm/kg	Percentage of initial fed-batch value
Manual Reading	Incubator Temperature	°C	Difference from standard temperature
Manual Reading	Shaker plate	rev/min	Percentage of initial fed-batch value
Manual Reading	Culture volume	mL	-
Manual Reading	Time	Days	Percentage of standard fed-batch reaction time

Appendix 3: Experiment 2 – Statistical Analysis of Glucose, Trace Metals, Amino Acids, and Vitamins

Response Peak Cell Density %

Actual by Predicted Plot



Summary of Fit

RSquare	0.9349
RSquare Adj	0.8047
Root Mean Square Error	5.6452
Mean of Response	213.34
Observations (or Sum Wgts)	19

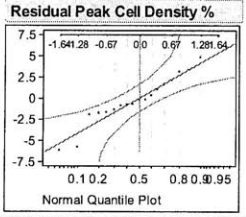
Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	12	2746.2	228.8	7.18
Error	6	191.2	31.9	Prob > F
C. Total	18	2937.4		0.0120

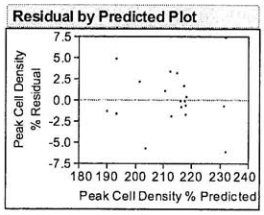
Sorted Parameter Estimates

Term	Estimate	Std Error	t Ratio	Prob>|t|
(Trace Metals-0.1)*(Trace Metals-0.1)	-2437	548.66	-4.44	0.0044
Initial Glucose (%)	0.1223	0.0322	3.80	0.0090
(Amino Acids-1.5)*(Initial Glucose (%)-370.466)	0.0681	0.0282	2.41	0.0523
Amino Acids	-2.016	1.2453	-1.62	0.1565
(Amino Acids-1.5)*(Vitamins-1)	1.5261	0.9851	1.55	0.1723
(Vitamins-1)*(Initial Glucose (%)-370.466)	0.0384	0.0315	1.22	0.2685
(Trace Metals-0.1)*(Vitamins-1)	-19.74	16.609	-1.19	0.2795
(Trace Metals-0.1)*(Amino Acids-1.5)	11.255	10.007	1.12	0.3037
(Trace Metals-0.1)*(Initial Glucose (%)-370.466)	0.31	0.344	0.90	0.4023
Vitamins	-1.13	1.714	-0.66	0.5341
Trace Metals	8.0613	15.764	0.51	0.6274
(Initial Glucose (%)-370.466)*(Initial Glucose (%)-370.466)	0.0005	0.0016	0.30	0.7739

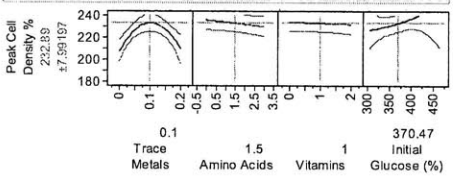
Residual Peak Cell Density %



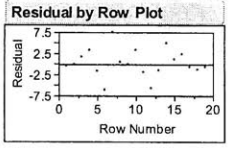
Residual by Predicted Plot



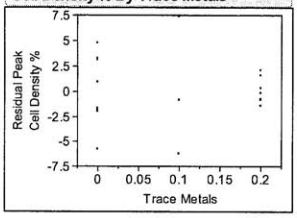
Prediction Profiler



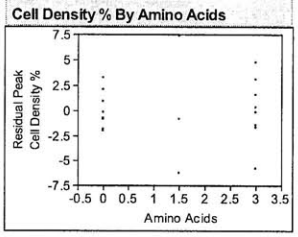
Residual by Row Plot



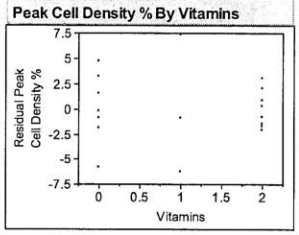
Bivariate Fit of Residual Peak Cell Density % By Trace Metals



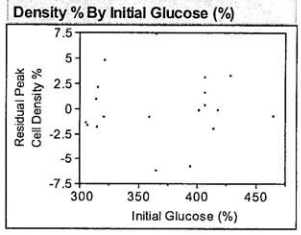
Bivariate Fit of Residual Peak Cell Density % By Amino Acids



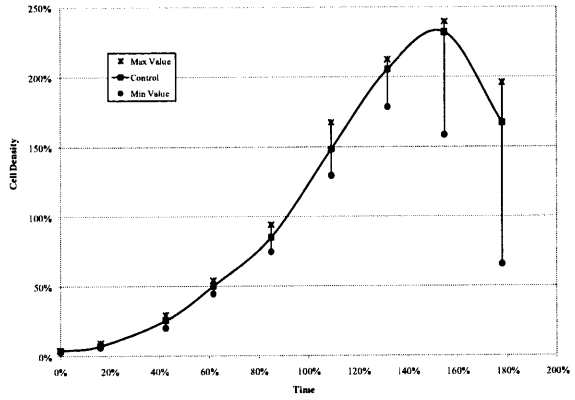
Bivariate Fit of Residual Peak Cell Density % By Vitamins



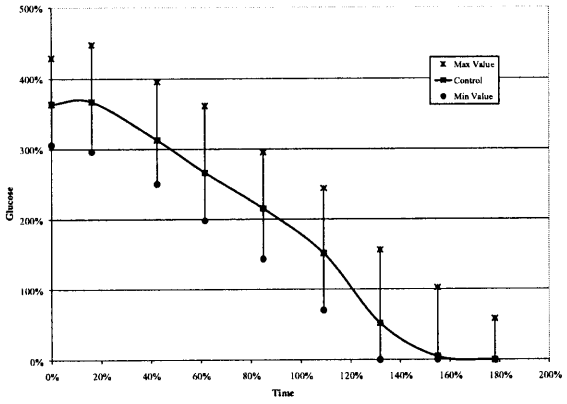
Bivariate Fit of Residual Peak Cell Density % By Initial Glucose (%)



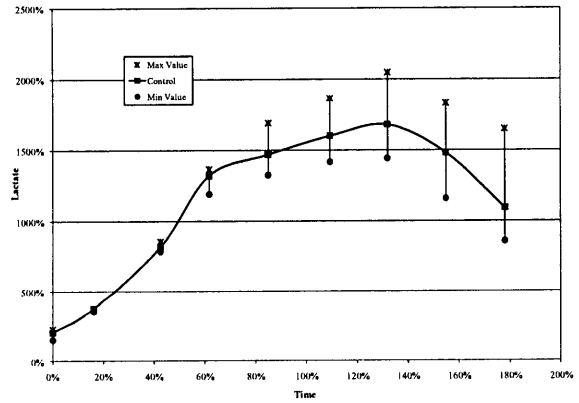
Appendix 4: Experiment 2 - Cell Density vs. Time



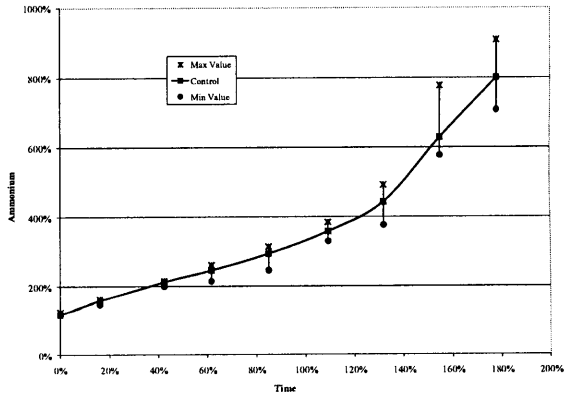
Appendix 5: Experiment 2 - Glucose vs. Time



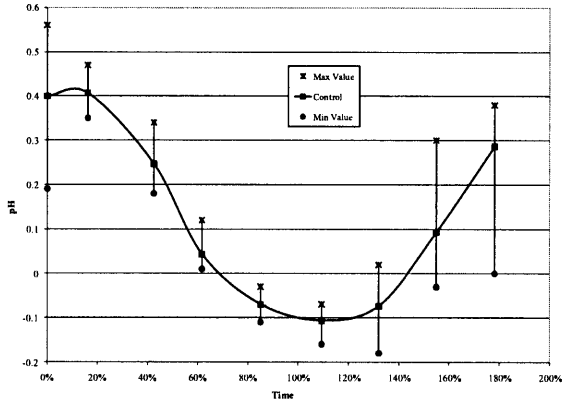
Appendix 6: Experiment 2 - Lactate vs. Time



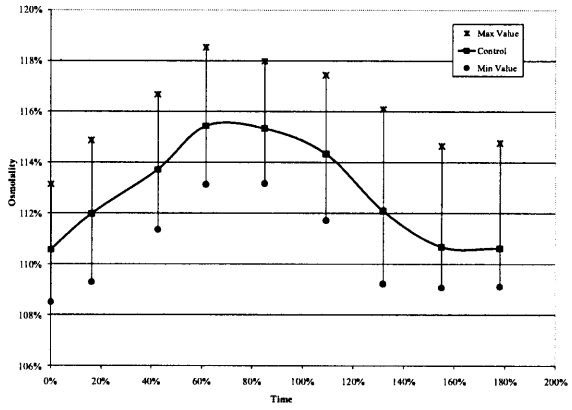
Appendix 7: Experiment 2 - Ammonium vs. Time



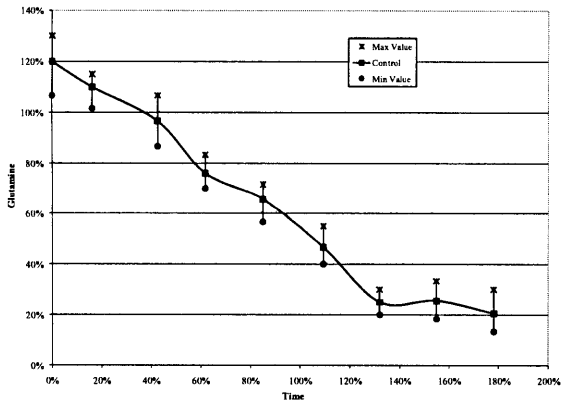
Appendix 8: Experiment 2 - pH vs. Time



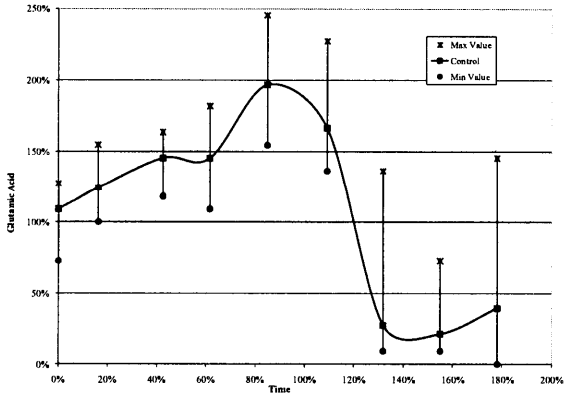
Appendix 9: Experiment 2 - Osmolality vs. Time



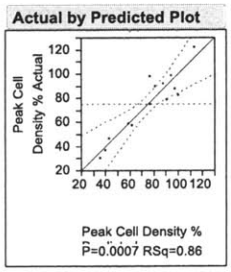
Appendix 10: Experiment 2 – Glutamine vs. Time



Appendix 11: Experiment 2 – Glutamic Acid vs. Time



Appendix 12: Experiment 3 – Statistical Analysis of pH, pO_2, Stir Speed, and Temperature



Summary of Fit

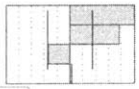
RSquare	0.858
RSquare Adj	0.795
Root Mean Square Error	11.88
Mean of Response	75.25
Observations (or Sum Wgts)	14

Analysis of Variance

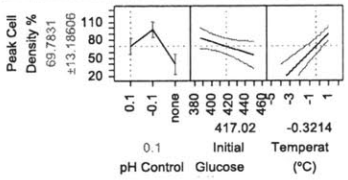
Source	DF	Sum of Squares	Mean Square	F Ratio
Model	4	7665.64	1916.41	13.5759
Error	9	1270.46	141.16	Prob > F
C. Total	13	8936.10		0.0007*

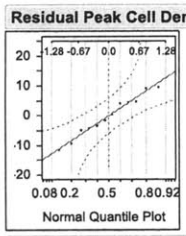
Sorted Parameter Estimates

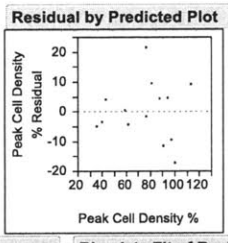
Term	Estimate	Std Error	t Ratio	Prob>|t|
Temperature (°C)	16.061	2.525	6.36	0.0001*
pH Control[-0.1]	28.271	5.757	4.91	0.0008*
Initial Glucose (%)	-0.445	0.205	-2.17	0.0584
pH Control[0.1]	0.691	4.886	0.14	0.8906

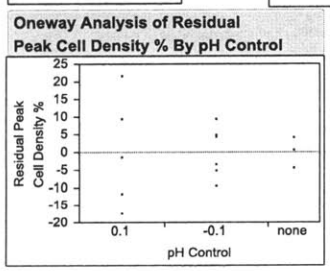


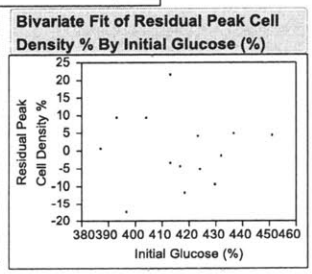
Prediction Profiler

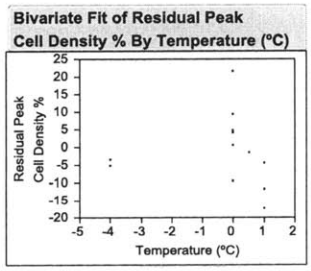






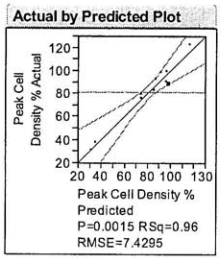






*All parameters not listed in the above analysis were removed because they did not significantly influence the model and thus does not influence cell growth

Appendix 13: Experiment 3 – Bioreactors with pH Control - Statistical Analysis of Temperature, pH, pO_2, and Stir Speed



Summary of Fit

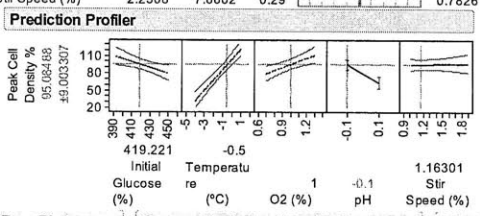
RSquare	0.9614
RSquare Adj	0.9229
Root Mean Square Error	7.4295
Mean of Response	80.979
Observations (or Sum Wgts)	11

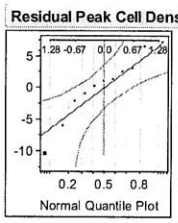
Analysis of Variance

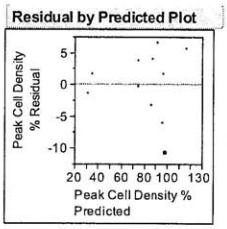
Source	DF	Sum of Squares	Mean Square	F Ratio
Model	5	6881.45	1376.29	24.9342
Error	5	275.984	55.20	Prob > F
C. Total	10	7157.43		0.0015

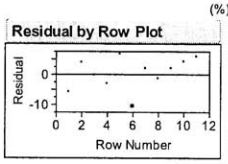
Sorted Parameter Estimates

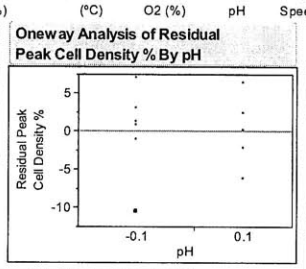
Term	Estimate	Std Error	t Ratio	Prob>|t|
Temperature (°C)	17.928	1.7202	10.42	0.0001
pH[-0.1]	15.517	2.9617	5.24	0.0034
O2 (%)	53.007	12.73	4.16	0.0088
Initial Glucose (%)	-0.504	0.1503	-3.36	0.0202
Stir Speed (%)	2.2308	7.6602	0.29	0.7826

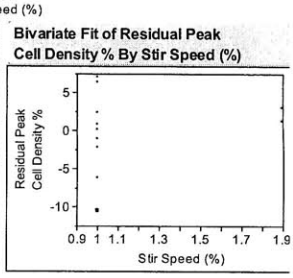


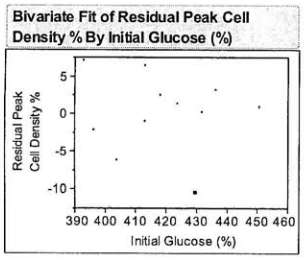


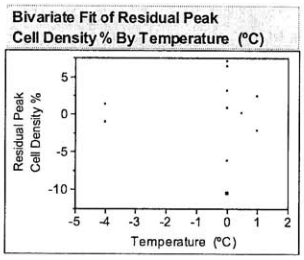


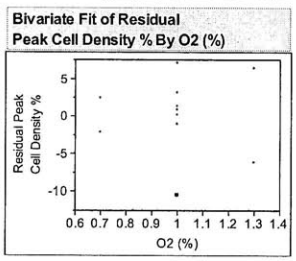




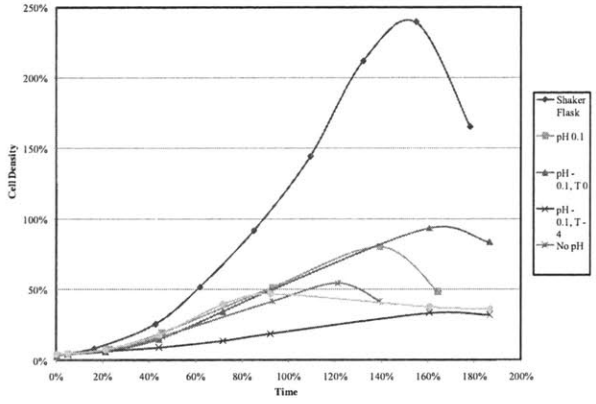




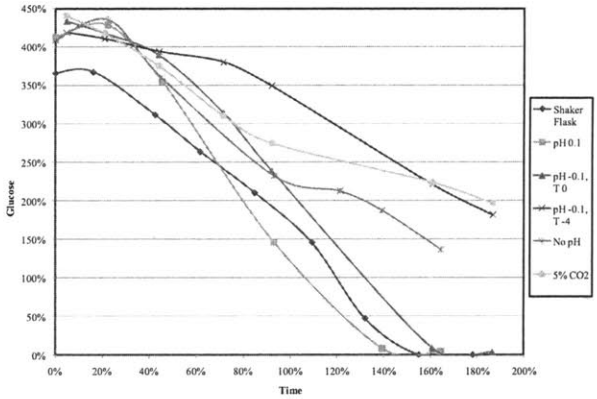




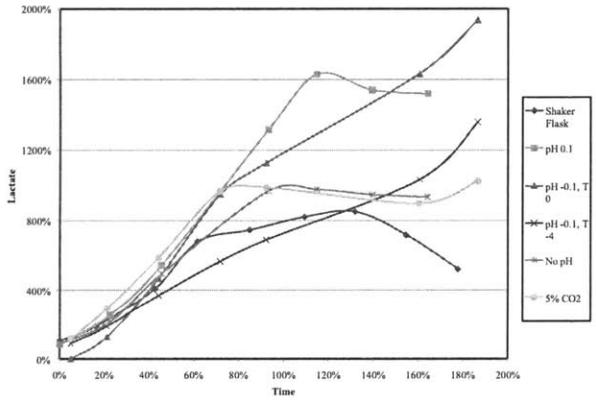
Appendix 14: Experiment 3 - Cell Density vs. Time



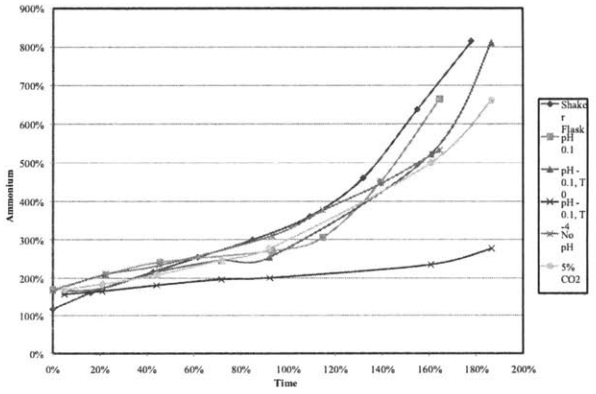
Appendix 15: Experiment 3 - Glucose vs. Time



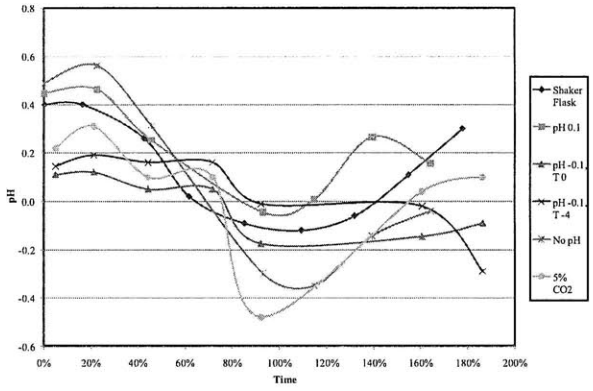
Appendix 16: Experiment 3 - Lactate vs. Time



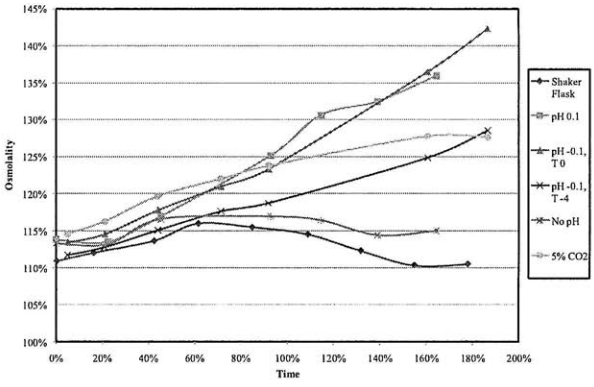
Appendix 17: Experiment 3 - Ammonium vs. Time



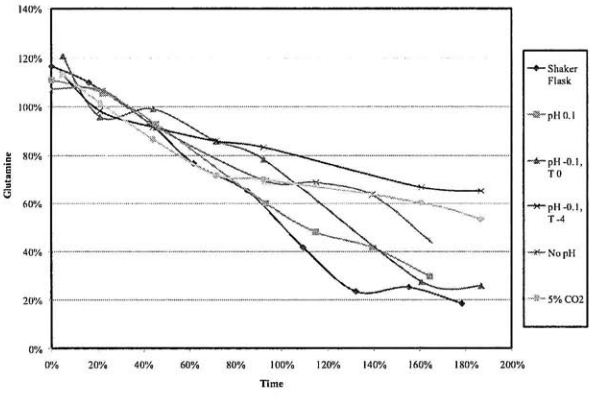
Appendix 18: Experiment 3 - pH vs. Time



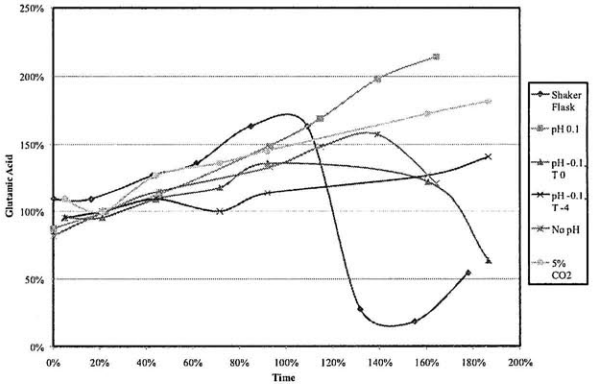
Appendix 19: Experiment 3 - Osmolality vs. Time



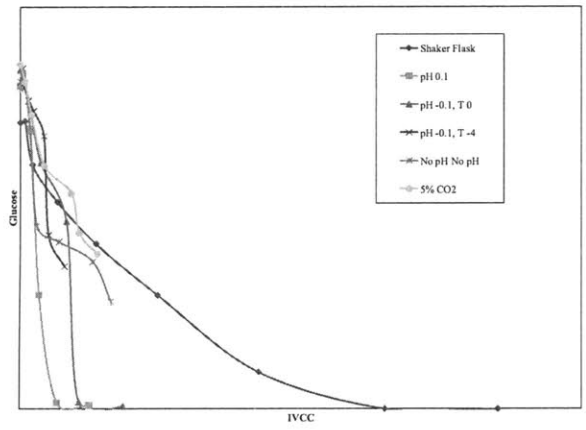
Appendix 20: Experiment 3 - Glutamine vs. Time



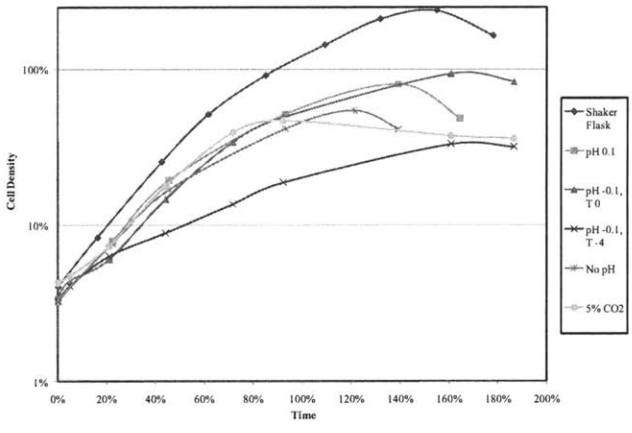
Appendix 21: Experiment 3 - Glutamic Acid vs. Time



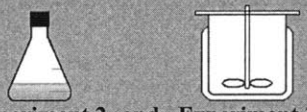
Appendix 22: Experiment 3 - Glutamic Acid vs. Time



Appendix 23: Experiment 3 - Glutamic Acid vs. Time



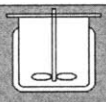
Appendix 24: Comparison of Shaker Flasks from Experiment 2 and Bioreactors from Experiment 3

	Discussion of Differences Between  **Experiment 2 and Experiment 3**
Cell Density	There is a clear difference between cell growth that occurs between day two and day three. Graphing cell growth on a log scale versus time shows that both experiments three phases of growth but the initial cell growth phases lasts for only two days with the bioreactors where it lasts for three days in the shaker flasks. The second growth phase, which lasts three days and is a lower growth rate than the first phase, and the plateau phase, where growth stops,

	seem to be similar between the two experiments.
Glucose	The same lot of glucose was used for the raw material for both experiments. The specific glucose chart graphs glucose versus the amount of cell produced for a given amount of time. As the trend line becomes more vertical it means that more glucose is consumed per cell and more horizontal means that less glucose is consumed. The specific glucose chart shows that the shaker flask samples have three distinct phases, which matches the cell growth chart. The bioreactor samples only have one phase of high glucose consumption.
Lactate	Lactate is one area where the shaker flasks and bioreactors seem to differ greatly. The lactate trend for the shaker flasks seems to follow the three growth phases with three days of significant lactate production during the initial growth phase, followed by four days of slow production during the second growth phase, and finally a decrease of lactate during the plateau phase. The lactate produced by shaker flasks levels off below 1,200%, which past experience shows that lactate concentration above this level can negatively impact the infection stage[18]. Lactate production for the bioreactors shows a similar pattern to shaker flask lactate production, but the different phases do not match the growth phases observed on the cell growth graph and the lactate production levels off at significantly higher value in the bioreactors than in the shaker flasks. Lactate, as well as in conjunction with ammonium, is known to negatively influence MDCK cell growth so this may require further exploration[19].
Ammonium	Ammonium production in the shaker flask and bioreactor experiments seems to follow a similar trend. The lower temperature bioreactors, which grew to a low cell density, have noticeably lower ammonium production which may be a factor of the lower temperature setting, the lower cell production, or some combination of both factors. This may require further exploration.
pH	While pH is controlled in a bioreactor, the actual value, as determined by an offline probe, does not always match the online measurement and the online probe must be recalibrated. The offline bioreactor measurements compared to the shaker flask measurements show similar ranges and trends.

Osmolality	The osmolality trend is also significantly different between the bioreactors with pH control and the shaker flasks and bioreactors without pH control. This is not surprising as the bioreactor pH is controlled with NaOH which increases the osmolality as it is added.
Glutamine	Glutamine consumption seems to be similar between the two experiments. The bioreactors that grew significantly less cells also consumed less glutamine, as is to be expected.
Glutamic Acid	Glutamic acid is production seems to be similar between the two systems. It rises during cell growth and then is fully consumed as the plateau growth phase is reached after which it begins to rise again.
CDM Age	The CDM used in both experiments came from the same production lot. The bioreactor experiments occurred 49 and 82 days later than the shaker flask experiments. While the CDM is stored at 4°C to reduce influence of aging, it is possible that the media composition has changed enough to influence cell growth. A look at the starting value for glutamine and ammonium shows that the later experiments have a lower glutamine concentration and higher ammonium concentration, which is not surprising as glutamine degradation produces ammonium[20].
PDL of Pre-Culture Cells	Cells pulled from the working cell bank for both experiments were 7-12 days removed from the freezing process and had been doubled between 20 and 30 times, which is well within the verified range. While there is a slight difference between the cell age of the cells used for the shaker flasks and the bioreactor, the difference is slight and unlikely to be influential.

Appendix 25: Comparison of Shaker Flask and Bioreactor Systems

	 **Shaker Flasks**	 **Bioreactors**	**Discussion of Differences Between Systems**

Temperature	Temperature of the incubator atmosphere is controlled and this maintains the temperature of the shaker flasks contents stored within the incubator. Direct measurement of the samples on the shaker plate indicates that the temperature of the cell culture is 0.5°C higher than the incubator set point. This higher temperature is caused by the shaker plate, which generates a small amount of heat.	Temperature is controlled by maintaining the temperature of water that is pumped into jacket surrounding the bioreactor.	The range of values tested in the bioreactors without achieving high-density cell growth indicates that temperature is not the limiting factor preventing high-density growth.
Oxygen	Oxygen is provided by the atmosphere and transfer between the air and the sample is maintained uniformly by vigorous agitation of the sample. Past experience places the above 75% dissolved O_2[21].	Oxygen is provided by the overlay gas and controlled by adding additional oxygen through a sparger at the bottom of the bioreactor directly below the internal stirrer. Percent dissolved O_2 is measured continuously by the Q+ system and maintained at the set point.	The range of values tested in the bioreactors without achieving high-density cell growth indicates that oxygen is not the limiting factor preventing high-density growth.
Agitation	Agitation is provided by an external orbital shaking device.	Agitation is provided by an internal stirrer.	The range of values tested in the bioreactors without achieving high-

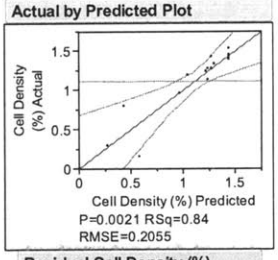
			density cell growth indicates that stir speed is not the limiting factor preventing high-density cell growth.
Carbon Dioxide	CO_2 is controlled to a constant 5% in the atmosphere within the incubator dissolved CO_2, which is transferred between the air and the sample, is maintained by vigorous agitation of the sample.	CO_2 is controlled by adding CO_2 gas to the gas overlay mixture when directed to by the Q+ system. Overlay gas CO_2 content is part of the pH control scheme in the Q+ system. CO_2 is added to the overlay gas, which then transfers to the sample, when the pH is above the set point.	There is difference between the way dissolved CO_2 is controlled in the two systems. The bioreactor experiment with high agitation and an overlay containing a constant 5% CO_2 was an attempt to replicate the settings of a shaker flask. The results of this experiment were not positive and additional study of dissolved CO_2 is required.
pH	The buffer system included in the CDM package provides some pH control, but otherwise pH is uncontrolled and changes as cell growth occurs.	The bioreactor system uses CO_2 gas and liquid NaOH to maintain a constant pH. The bioreactor pH sensor has to be recalibrated when the offline measurement does not match the online measurement.	Experimental data shows that pH is an important parameter for cell growth but turning the pH control system off in the bioreactor did not promote cell growth. When the bioreactor pH control system is turned off the only CO_2 provided to the system is atmospheric CO_2 provided through the gas overlay. It is possible that when

			the pH control system was turned off, CO_2 was exhausted and this limitation inhibited cell growth.
Surface Area to Liquid Volume Ratio	Oxygen and CO_2 is controlled in a shaker flask by reaching equilibrium with the atmosphere within the incubator. This exchange between the sample and the atmosphere occurs through the surface area of the sample. The surface area to liquid volume ratio in a shaker flask is 0.5 cm^2/mL.	Oxygen is controlled through sparging so it is not influenced by the surface area to liquid volume ratio, but CO_2 is controlled through overlay gas so it is influenced. The surface area to liquid volume ratio in a shaker flask is 0.4 cm^2/mL.	This difference between these ratios may explain why a bioreactor is not able to get as much CO_2 transferred into the liquid from the overlay gas, if that were an issue. More exploration on this is needed.
Filling Procedure	Shaker flasks are filled by first adding warmed media and glucose to a flask. Then, a sample of cells is added, the mixture is sampled, and the flask is immediately placed into the incubator.	Bioreactors are filled by first adding warmed media and glucose to a vessel. Then, a sample of cells is added, the mixture is sampled, and the contents are immediately transferred to the vessel.	There is no difference between the steps taken to generate the samples or the time required to prepare each sample.
Vessel Shape	Shaker flasks are Erlenmeyer flasks and thus shaped like an upside down cone. The vessels also have ridges along the inside of the bottom to act as	Bioreactors are cylindrical shaped with an internal top-stirrer placed in the center of the vessel.	While the shape of the vessel is unlikely to be a significant factor, there is a slight difference that can be further explored.

	baffles and aid in mixing.		
Vessel Material	Shaker flasks are made out of polycarbonate.	Bioreactors are made out of glass.	While the material of the vessel is unlikely to be a significant factor, there is a slight difference that can be further explored.
Light Exposure	Shaker flasks are kept in a closed incubator with no exposure to light.	The glass bioreactors are exposed to fluorescent lighting during work hours.	Past experience has shown that the media and cell line are not light sensitive, but this is a difference between the two systems that can be further explored.

Appendix 26: Experiment 4 - Statistical Analysis of Vessel, Material, and Time Pulled from Bioreactor

Actual by Predicted Plot



Summary of Fit

RSquare	0.8414
RSquare Adj	0.7533
Root Mean Square Error	0.2055
Mean of Response	1.1112
Observations (or Sum Wgts)	15

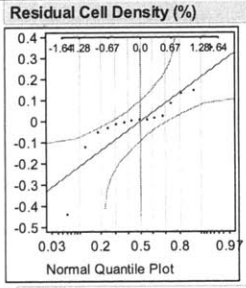
Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	5	2.0162	0.4032	9.5498
Error	9	0.3800	0.0422	Prob > F
C. Total	14	2.3963		0.002

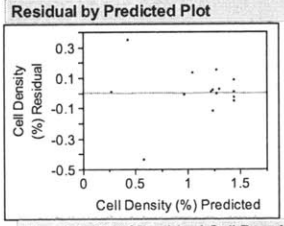
Sorted Parameter Estimates

Term	Estimate	Std Error	t Ratio	Prob>|t|
pH[-0.1]	0.3798	0.149	2.55	0.0312
Surface Area	-1.278	0.5136	-2.49	0.0346
Vessel Material[Glass]	-0.195	0.0928	-2.10	0.0653
Agitation Method[External Shake]	0.2965	0.1433	2.07	0.0685
Initial Cell Density (% Initial)	0.5392	0.4281	1.26	0.2395

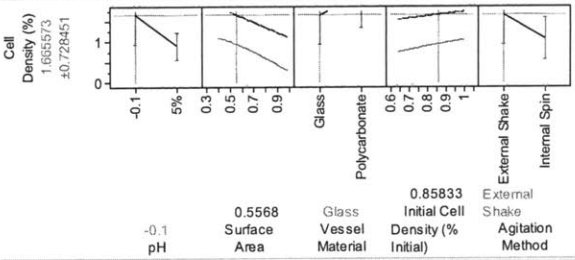
Residual Cell Density (%)



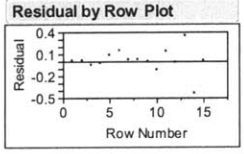
Residual by Predicted Plot



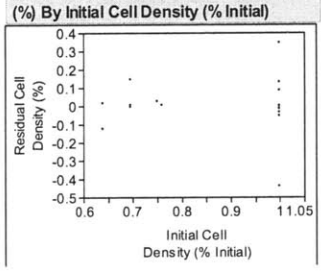
Prediction Profiler



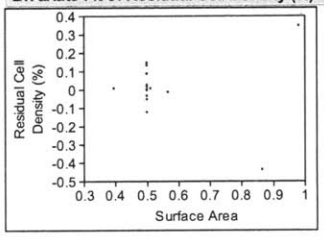
Residual by Row Plot



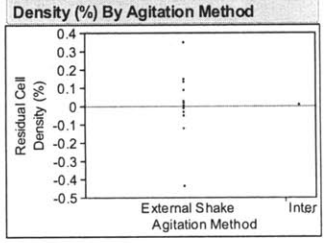
Bivariate Fit of Residual Cell Density (%) By Initial Cell Density (% Initial)



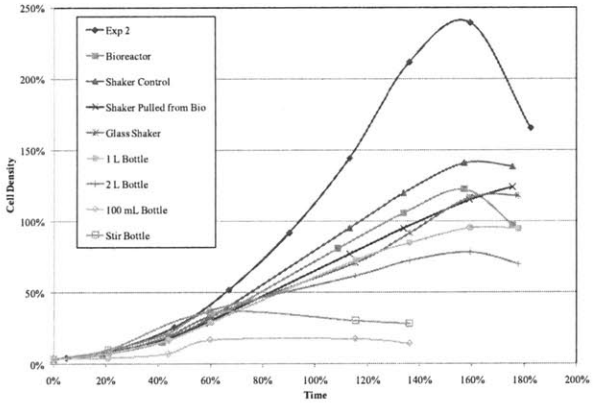
Bivariate Fit of Residual Cell Density (%) By Surface Area



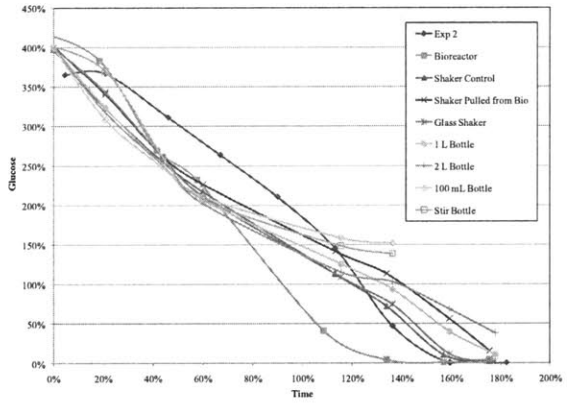
Oneway Analysis of Residual Cell Density (%) By Agitation Method



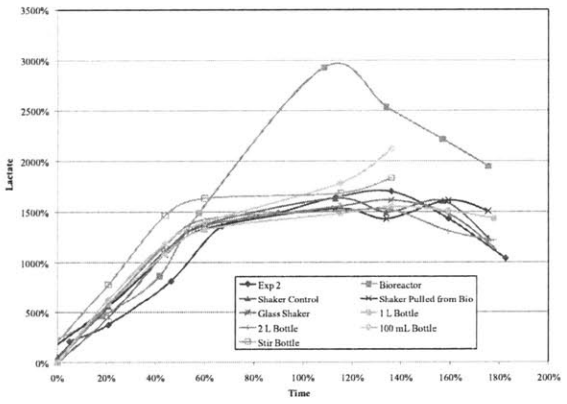
Appendix 27: Experiment 4 – Cell Density vs. Time



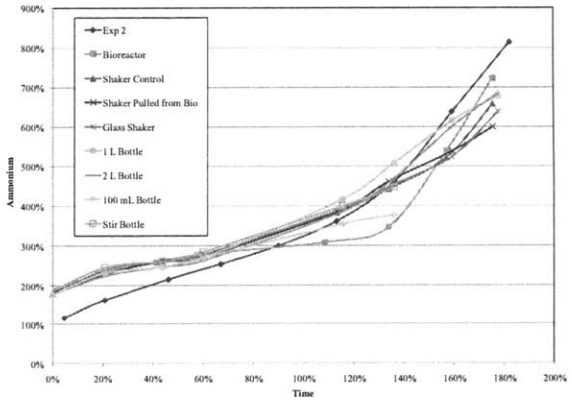
Appendix 28: Experiment 4 – Glucose vs. Time



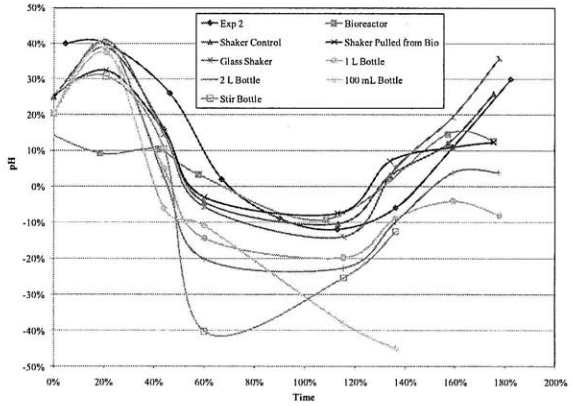
Appendix 29: Experiment 4 – Lactate vs. Time



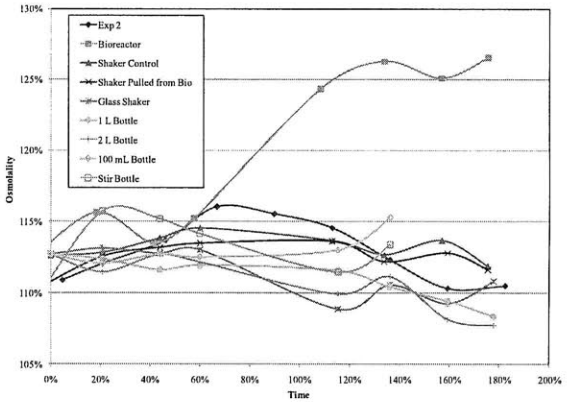
Appendix 30: Experiment 4 – Ammonium vs. Time



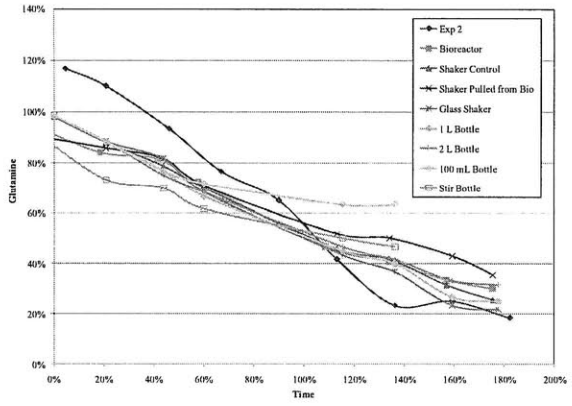
Appendix 31: Experiment 4 – pH vs. Time



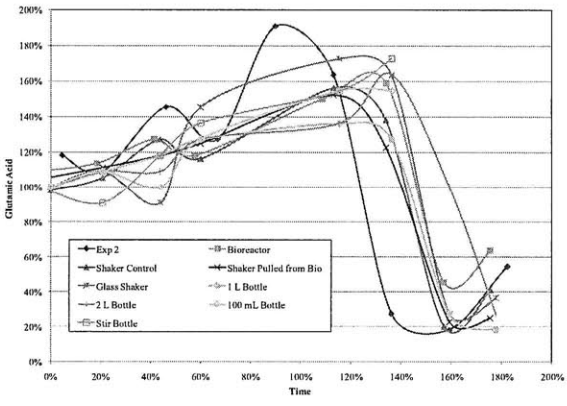
Appendix 32: Experiment 4 – Osmolality vs. Time



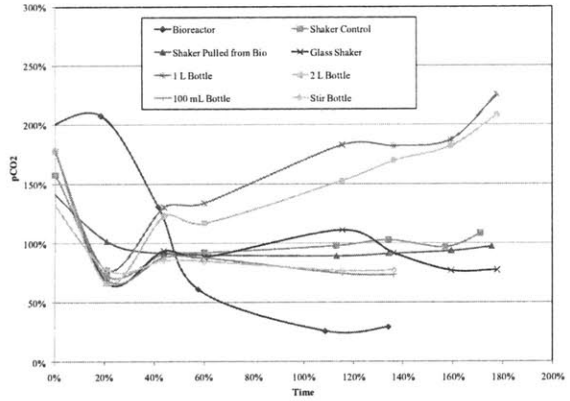
Appendix 33: Experiment 4 – Glutamine vs. Time



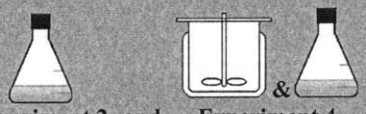
Appendix 34: Experiment 4 – Glutamic Acid vs. Time



Appendix 35: Experiment 4 – pCO_2 vs. Time



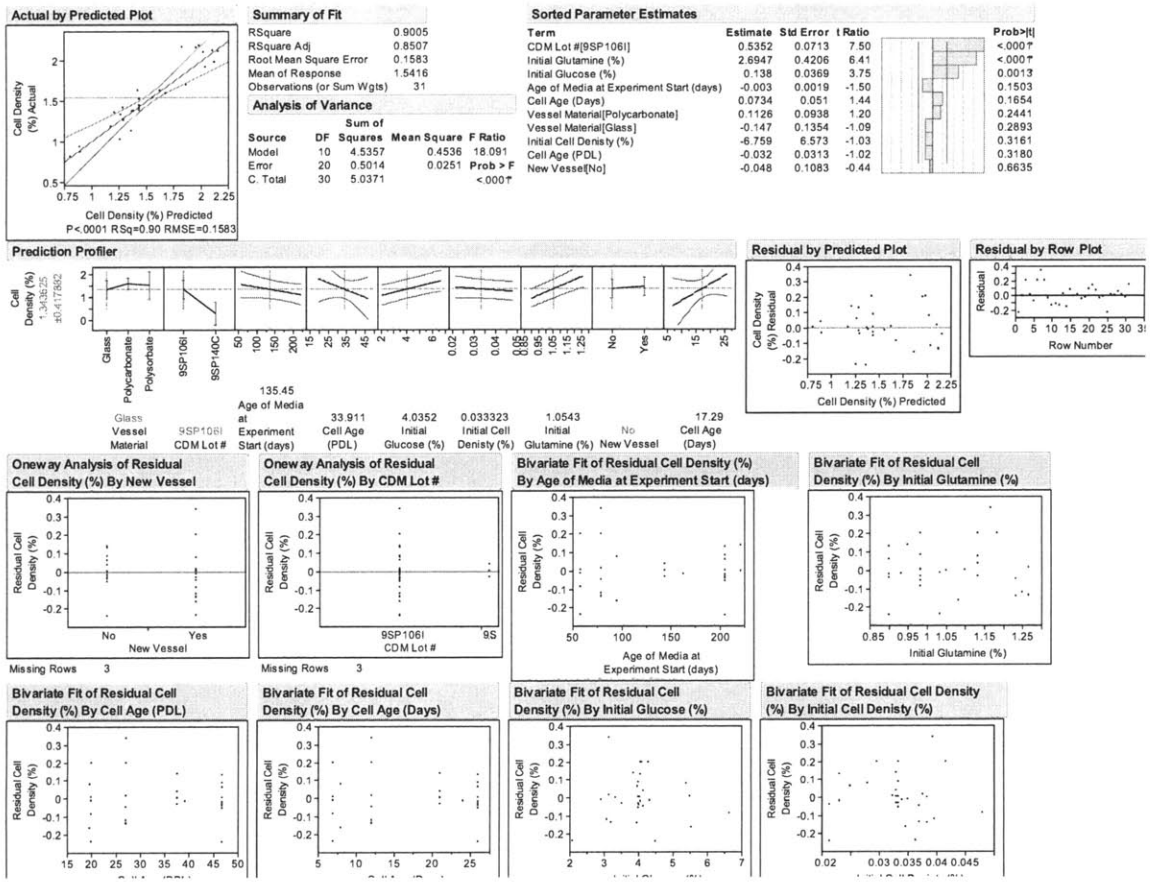
Appendix 36: Comparison of Shaker Flasks from Experiment 2 and Experiment 4

	Discussion of Differences Between  **Experiment 2 and Experiment 4**
Cell Density	Clearly the samples from Experiment 4 did not grow as well as the samples in Experiment 2. Again, the separation between the experiments seems to occur between a time of 40% and 80%.
Glucose	The graph of glucose concentration versus time shows that despite the difference in total cells grown, samples from both Experiment 2 and Experiment 4 run out of glucose at similar points in time which indicates that Experiment 4 samples consumed glucose less efficiently than samples from Experiment 2.

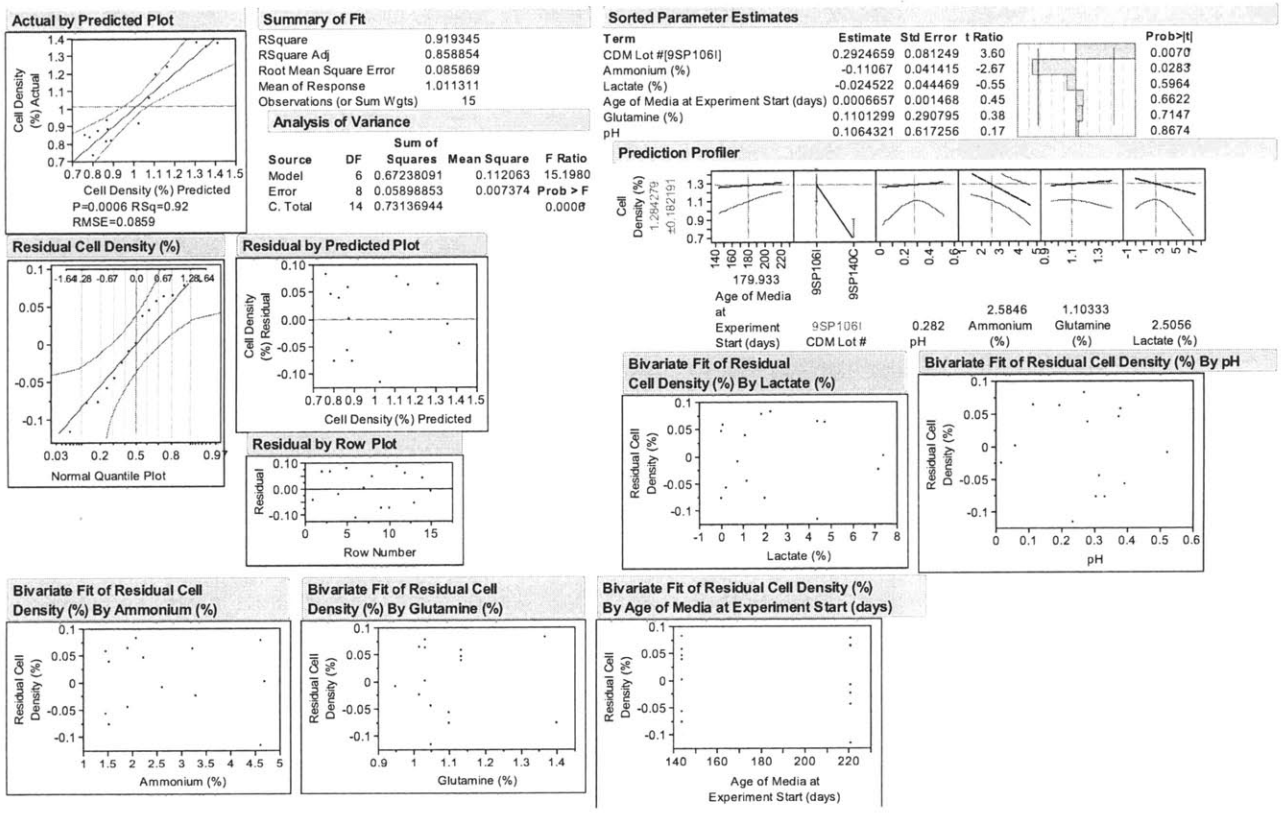
Lactate	Aside from the bioreactors, all samples from Experiment 4 display a similar lactate concentration trend to the samples from Experiment 2.
Ammonium	Ammonium is similar to glutamine in that the starting concentration levels are different between Experiment 2 and Experiment 4. Experiment 4 starting levels are higher than starting levels for Experiment 2, which makes sense as the degradation of glucose produces ammonium. Aside from the starting levels, ammonium trends between Experiment 2 and Experiment 4 are similar, thought the difference in starting concentration may be significant as ammonium is known to inhibit cell growth[22].
pH	pH for a majority of the flasks from Experiment 4 appears to follow the same trend as pH from Experiment 2. The only serious deviations are with the controlled bioreactors and the spinner flask and 100 mL bottle.
Osmolality	Aside from the bioreactors, all samples from Experiment 4 display a similar osmolality concentration trend to the samples from Experiment 2.
Glutamine	Glutamine concentration in Experiment 2 starts ~16% higher than the glutamine concentration in Experiment 4. This difference in starting value is likely due to the age of the media as glutamine degrades over time even when kept in cold temperature storage. Aside from the difference in starting concentrations the glutamine trend between Experiment 2 and Experiment 4 appear relatively similar.
Glutamic Acid	Glutamic acid follows a similar trend between Experiment 2 and Experiment 4.
CDM Age	The same lot of CDM was used for both Experiment 2 and Experiment 4. At the time Experiment 2 was conducted the media used was 79 days old and at the time Experiment 4 was conducted the media was 205 days old. While the media was stored in near freezing temperature during this time period and is within the acceptable usage period that this process has been verified, this is a difference that should be investigate further.
PDL of Pre-	Cells used for Experiment 4 were 25 days removed from the freezing process and had been doubled 45 times

Culture Cells	while cells used for Experiment 2 were 7 days removed and had been doubled 20 times. Both of these values are well within the limits of the Novartis process for this cell line, so this difference is not likely to be a significant factor. Also, the statistical analysis seen in Appendix 37: Statistical Analysis of All Blank Shaker Flasks shows that pre-culture cell age is not an influential factor.

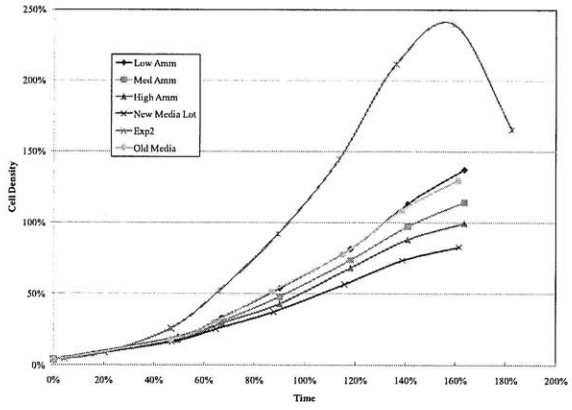
Appendix 37: Statistical Analysis of All Blank Shaker Flasks



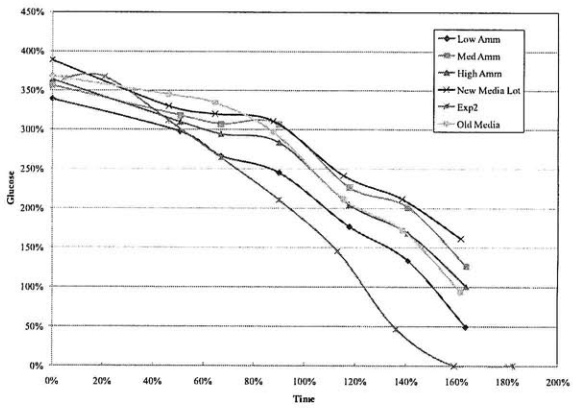
Appendix 38: Experiment 5 - Statistical Analysis of Glutamine, Lactate, Ammonium, and Media Age



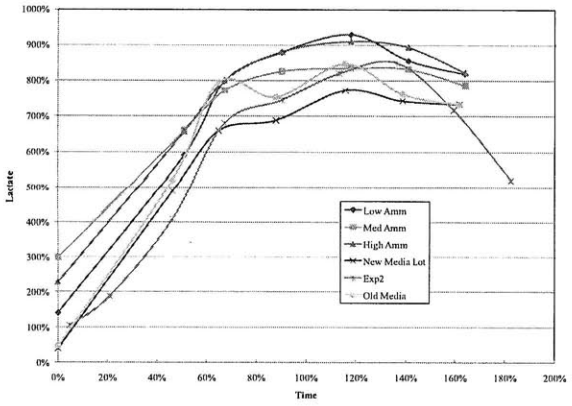
Appendix 39: Experiment 5 – Cell Density vs. Time



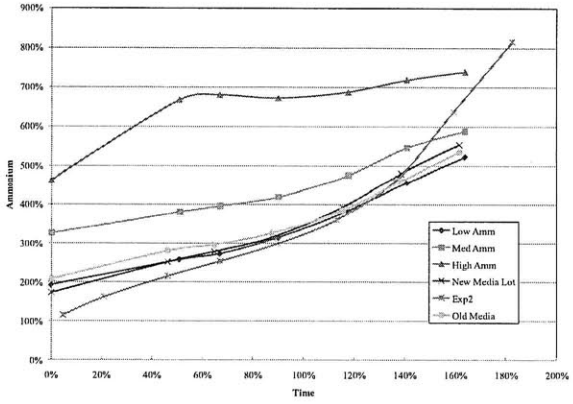
Appendix 40: Experiment 5 – Glucose vs. Time



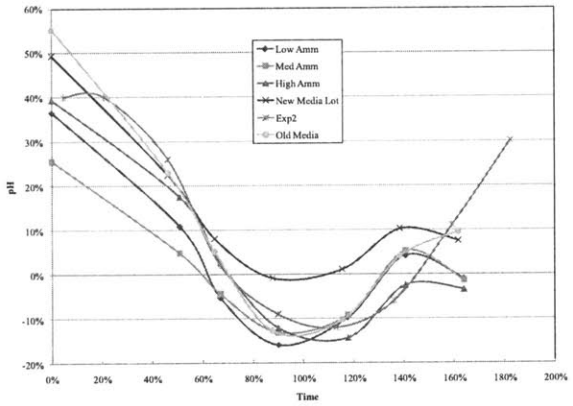
Appendix 41: Experiment 5 – Lactate vs. Time



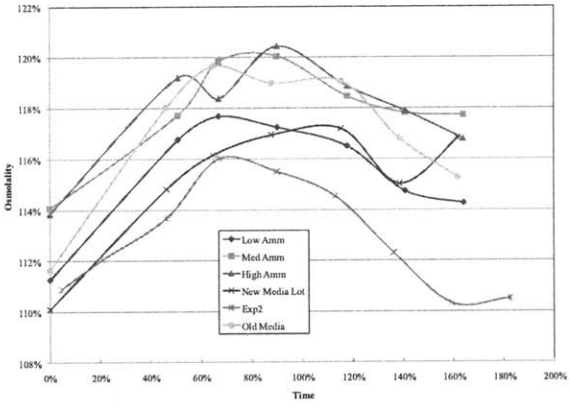
Appendix 42: Experiment 5 – Ammonium vs. Time



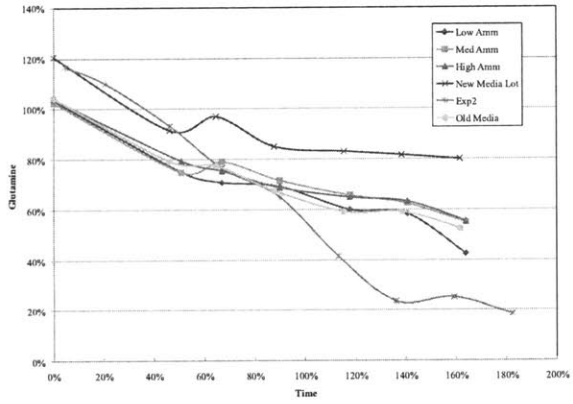
Appendix 43: Experiment 5 – pH vs. Time



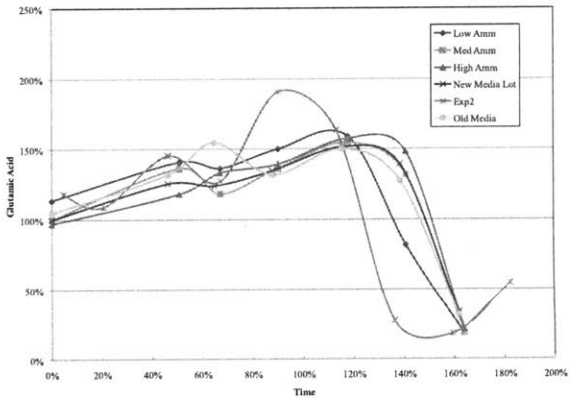
Appendix 44: Experiment 5 – Osmolality vs. Time



Appendix 45: Experiment 5 – Glutamine vs. Time



Appendix 46: Experiment 5 – Glutamic Acid vs. Time



Appendix 47: Discussion of Results from Experiment 5

	Discussion of Differences Between
Cell Density	Again, the samples did not grow as well as Experiment 2 and the separation between the experiments seems to occur between a time of 40% and 80%. Surprisingly, there is a substantial difference between the new media and old media, where we would have expected identical results.
Glucose	The samples in Experiment 2 consumed glucose faster than the samples in Experiment 5 which is expected as fewer cells grew in Experiment 5. No major difference is observed between the samples of Experiment 5.
Lactate	While lactate levels started differently, all samples followed a similar trend and leveled off at a similar value. The analysis seen in Appendix 38: Experiment 5 - Statistical Analysis of Glutamine, Lactate, Ammonium, and Media Age further confirms that the starting lactate level did not impact cell growth.
Ammonium	Ammonium production and cell growth show a distinct difference between samples that started at a higher ammonium value and the samples that started at a lower ammonium value. The importance of the initial ammonium value is confirmed in the linear regression. Unfortunately, the ammonium level of the old and new media are near identical so initial ammonium level does not explain the difference between the two. It is possible that a higher starting ammonium level is prohibiting cells from Experiment 5 from reaching levels achieved in Experiment 2.
pH	pH follows the same trend as pH from Experiment 2 and there was no major difference between the samples in Experiment 5.
Osmolality	Osmolality follows the same trend as osmolality from Experiment 2 and there was no major difference between the samples in Experiment 5.
Glutamine	Glutamine consumption is clearly tied to cell growth but it does not to be influential in encouraging cell growth.

	The higher glutamine samples, from the new media lot, do not grow any better than the lower glutamine samples.
Glutamic Acid	Glutamic acid follows a similar trend between Experiment 2 and Experiment 5 and within the samples of Experiment 5.
CDM Age	A different lot was found to simulate new media but the results opposite of what previous experiment predicted. The older media grew better than the newer media, and the newer media failed to reach even 100% cell density. This implied that something other than media age is inhibiting cell growth.

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